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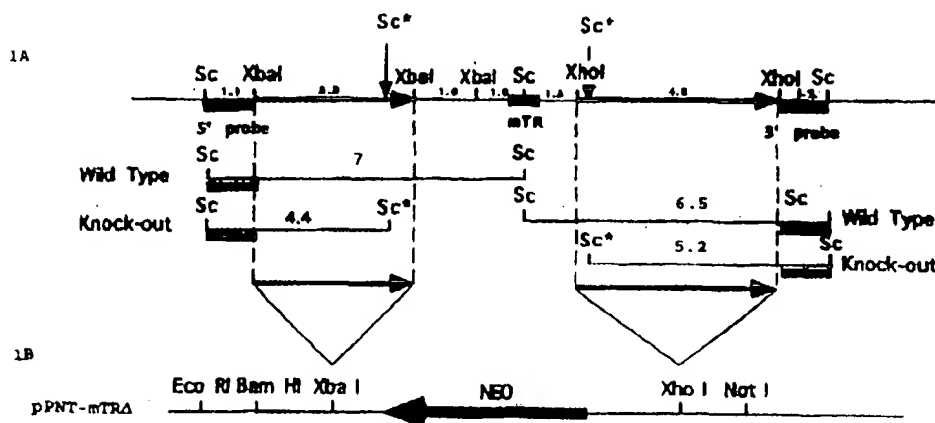
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TARGETING CONSTRUCT FOR KNOCKING OUT MOUSE TELOMERASE RNA COMPONENT



* Site-directed mutagenesis

(57) Abstract

Transgenic nonhuman organisms, such as transgenic mice, with altered expression of telomerase are provided. Methods of using these organisms, including methods of detecting compounds that affect the expression of telomerase are also disclosed.

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TRANSGENIC ORGANISMS WITH ALTERED TELOMERASE ACTIVITY

Background of the Invention

Telomerase is a ribonucleoprotein DNA polymerase that adds nucleotide sequence repeats to the telomeres of chromosomes as cells divide. Telomerases are comprised of essential RNA and protein components. Blasco, M.A., et al. (1995) *Science* 269:1267-1270; Feng, J., et al. (1995) *Science* 269:1236-1241; Collins, K., et al. (1995) *Cell* 81:677-686. Without telomerase, the telomeres of chromosomes shorten with each replication until they reach a critical length at which chromosome stability is affected.

Studies of telomerase suggest that the telomerase enzyme may be a new target for cancer therapy and a key to aging and the finite lifespan of somatic cells. Harley, C., et al. (1990) *Nature* 345:458-460. Kim, et al. (1994) *Science* 266:2011-2015, were not able to detect telomerase in a large number of primary cell lines and primary human tissues. When the number of telomeric simple sequence repeats reaches a critical minimum size, these cells become senescent. Harley, C., *supra*; Counter, et al. (1992) *EMBO J.* 11:1921-1929. Unlike most somatic cells, germline cells express telomerase and maintain telomere length throughout cell divisions.

Short telomeres are also found in immortalized cells, including human tumor cell lines. In contrast to normal human somatic cells, however, cancer cells from tissue culture and those taken directly from tumors contain detectable telomerase activity, suggesting the telomere length is maintained so these cells can divide indefinitely. Counter, et al. (1994) *PNAS* 91:2900-2904; Kim, et al., *supra*. These findings suggest that targeting telomerase may be an effective cancer treatment. Harley,

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et al. (1994) *Cold Spring Harbor Lab Symposium on Quantitative Biology*, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. Because telomerase plays a key role in aging and human disorders, it is essential to understand
5 how telomerase functions.

Summary of the Invention

This invention provides nonhuman transgenic organisms in which telomerase activity is altered. Organisms with one altered gene are heterozygous for the alteration;
10 whereas, organisms in which the alteration occurs in both alleles are homozygous for the alteration.

In one embodiment, telomerase activity is reduced or eliminated in the nonhuman transgenic organisms because the gene or genes encoding one or more telomerase components is
15 "knocked out" (i.e., deleted or otherwise disabled), with the result that telomerase activity is reduced or eliminated.

In a second embodiment, nonhuman transgenic organisms contain nucleic acid constructs which induce telomerase
20 activity by turning on endogenous genes encoding telomerase RNA and protein components that are normally silent, or through insertion of one or more nucleic acid constructs comprising DNA or RNA encoding telomerase RNA and/or protein components into the genome of an organism, in which
25 they are expressed. Alternatively, an exogenous DNA construct, such as a promoter gene, is inserted into the genome of an organism and alters the normal transcription or functioning of endogenous telomerase. This promoter can be an inducible promoter, which is inserted and integrated
30 into the genome of the organism so that endogenous or exogenous genes encoding telomerase are turned on or off at particular times and in selected tissues.

In a third embodiment, a nonhuman transgenic organism is provided wherein an endogenous telomerase component gene

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is replaced, at least in part, with a telomerase component gene from another species or a telomerase component gene from another species which has been altered, thus producing an organism with a chimeric gene. The replacement can
5 occur on both endogenous genes, resulting in an organism that is homozygous for the exogenous chimeric gene and is not capable of expressing any telomerase gene at that genomic location or is only capable of expressing the novel nucleotide sequence or transgene.

10 In another aspect, the invention provides a rodent, such as a mouse in which expression of the gene encoding a telomerase component is altered. In one embodiment, the endogenous gene is removed, partially or completely and, as a result, telomerase activity is reduced or absent. The
15 rodent can be heterozygous or homozygous for the alteration. In a second embodiment, telomerase activity is induced by providing a DNA construct which turns on a normally silent endogenous gene. In a third embodiment, telomerase expression is altered by insertion, into the
20 genome of the rodent, of a nucleic acid sequence that replaces all or a part of endogenous DNA encoding a telomerase RNA or protein component, the result of which is a chimeric telomerase molecule.

The nucleic acid sequence which replaces the
25 endogenous gene encoding a telomerase component or a portion thereof can be a novel DNA sequence, a portion of the telomerase component gene, a marker gene, a promoter or other regulatory sequence, or a combination of these sequences.

30 The invention further provides constructs, particularly DNA constructs, useful for producing the transgenic nonhuman organisms, such as transgenic mice, described herein. Also provided are nucleic acid probes which can be used to distinguish DNA of a wildtype
35 (naturally-occurring) organism from DNA of an organism in

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which a portion of an endogenous telomerase component gene has been replaced with an exogenous DNA sequence. Further, nucleic acid constructs of transgenic unicellular eukaryotes such as *Tetrahymena sp.*, or in transfected
5 prokaryotes are disclosed which are useful for production of telomerase or telomerase components.

In another aspect, this invention provides embryonic stem cells, somatic cells and tissues of a nonhuman organism which contain one or more copies of the nucleic
10 acid constructs described herein. This includes cells and tissues comprising knockout constructs or constructs which induce telomerase expression. The transgenic organisms can be used as a source of cells for cell culture.

In a further aspect, the invention provides a method
15 of identifying a drug for stimulating telomerase activity in a transgenic nonhuman organism, such as a mouse, with reduced telomerase activity. Alternatively, compounds that inhibit telomerase activity can be identified and/or tested for toxicity using a transgenic nonhuman organism to
20 determine if telomerase can be inhibited or suppressed without detrimental effects to the organism. The drug can be administered to the organism and a sample of cells or tissues from the organism can be assayed for telomerase activity and for toxic side effects.

25 The invention further provides a method of using a transgenic organism or cells or tissues from the organism or its descendants to identify the control elements of immortal cells, such as cancer cells, or to identify the controlling factors in agents designated for anti-tumor or
30 anti-aging purposes. In addition, agents that stimulate or restrict these phenomena can be identified and developed for prophylactic or therapeutic applications through the use of such transgenic organisms and their descendants.

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Brief Description of the Drawings

- Figures 1A and 1B diagram the targeting construct for knocking out the mouse telomerase RNA component. Figure 1A diagrams the portion of the wildtype mouse genome which includes the mouse telomerase RNA component (mTR). Plasmid pPNT-mTRΔ (Figure 1B) shows the vector used to replace the endogenous 3.9 kb chromosomal segment including the mouse gene for the telomerase RNA component, with a neomycin resistance (NEO) gene.
- Figure 2 is a restriction map of the genomic mTR gene. Figure 3 shows the XhoI and XbaI fragments with introduced SacI (Sc*) restriction sites.
- Figure 4 shows the XhoI and XbaI genomic fragments with inserted SacI* (Sc*) sites cloned into the Bluescript SK- and KS+ plasmids and into pPNT-mTRΔ.
- Figure 5 is the nucleotide sequence (SEQ ID NO:1) of DNA encoding the human telomerase RNA component with the telomeric repeat template underlined and the start (ST) and end (STP) of transcription sites marked.
- Figure 6 is the nucleotide sequence (SEQ ID NO:2) of DNA encoding the mouse telomerase RNA component with the telomeric repeat template underlined.
- Figure 7A-7B is a comparison of DNA encoding the human (hTR), mouse (mTR), rat (rTR) (SEQ ID NO:3), hamster (cTR) (SEQ ID NO:4), and bovine (bTR) (SEQ ID NO:5) telomerase RNA components showing the conserved sequences between the molecules.
- Figure 8 is the nucleotide sequence (SEQ ID NO:6) of the *Tetrahymena* 80 kD telomerase protein component gene. The nucleotide sequence is derived from genomic and cDNA clones.
- Figure 9 is the amino acid sequence (SEQ ID NO:7) of the *Tetrahymena* 80 kD protein component deduced from the nucleotide sequence shown in Figure 8.
- Figure 10 is the nucleotide sequence (SEQ ID NO:8) of

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the *Tetrahymena* 95 kD telomerase protein component gene.

Figure 11 is the amino acid sequence (SEQ ID NO:9) of the *Tetrahymena* 95 kD protein component deduced from the nucleotide sequence shown in Figure 10.

5 Figure 12 is the nucleotide sequence of a genetically-engineered p80 telomerase protein component gene.

Figure 13 is the nucleotide sequence of a genetically-engineered p95 telomerase protein component gene.

Detailed Description of the Invention

10 This invention provides transgenic nonhuman organisms, such as transgenic mice, in which telomerase activity is altered. In one embodiment, telomerase activity is reduced or absent because one or more of the endogenous genes encoding a telomerase component is lacking or does not
15 encode a functional telomerase component. In this example, all or a portion of the endogenous or wildtype gene has been replaced with an exogenous nucleic acid sequence, i.e., a sequence not normally found in the corresponding wildtype species. A knockout organism is one in which both
20 of the endogenous genes for a telomerase component have been completely disabled. Telomerase activity is absent in a knockout organism; i.e., telomerase activity is absent in somatic and germline cells compared to a wildtype organism of the same species. The transgenic organisms described
25 include heterozygous and homozygous organisms. Heterozygotes include transgenic organisms in which one of the two copies of a telomerase component gene is altered and telomerase activity is the same or is reduced in somatic or germline cells compared to a wildtype organism
30 of the same species.

The invention further comprises cells or tissues of transgenic nonhuman organisms wherein telomerase expression is otherwise altered. For example, transgenic nonhuman organisms are provided in which telomerase activity is

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activated through the insertion of one or more genes encoding a telomerase component. Alternatively, a DNA construct is incorporated into the genome of the organism and selectively regulates genes encoding telomerase so that
5 telomerase activity can be induced or inhibited at particular times or in selected tissues.

Transgenic nonhuman organisms are also provided wherein the organism contains all or part of an exogenous DNA sequence encoding a telomerase component gene from
10 another species. The resulting telomerase molecules comprise at least part of an exogenous telomerase component which replaces the corresponding nucleotide sequence of the endogenous gene.

As used herein, the term "transgenic nonhuman
15 organism" means organisms which result from alteration of one or more endogenous genes encoding a telomerase component (founder organisms) and all subsequent generations. The term "descendants" refers to any and all future generations derived or descending from a "founder"
20 transgenic organism, e.g., an organism containing an exogenous construct as part of its genomic DNA and able to transmit this construct through its germ cells. Thus, descendants of any successive generation are included herein if the descendants contain the alteration or
25 transgene as part of their genome.

The organisms referred to in this application include all vertebrate and invertebrate multicellular organisms described in the kingdom Animalia, unicellular and multicellular fungi, and all animal-like protists. The
30 term "animal-like protists" includes all unicellular eukaryotes that are absorptive or ingestive heterotrophs, including, e.g., *Tetrahymena* sp., *Amoeba*, sp., and parasitic protozoa such as *Trichomonas* sp., *Giardia* sp., *Entamoeba* sp., *Plasmodium* sp. and *Leishmania* sp.. See,
35 e.g., Lee, J.J., et al. (eds.) *An Illustrated Guide to the*

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Protozoa, Society of Protozoologists, Lawrence KS, 1985. All references to nonhuman organisms are meant to include species of Animalia, Fungi, and animal-like species of the Protista, except in instances where it is obvious that a multicellular animal is intended (e.g., references to blood, vertebrate, ES cells, an organ, tumor, etc.).

Although mice homozygous for the deletion of a telomerase component (homozygous null) are known (see Example 6), cells or tissues can be derived from any stage of development of an organism homozygous for a telomerase deletion, such as a homozygous embryo, fetus, or immature animal. The cells can be cultured, using standard cell or tissue culture techniques, and then used to study the functioning of cells in the presence of various agents and can function as implants in organisms with normal telomerase activity.

Implants can be especially useful when inserted into nude mice which are prone to tumor formation. The mice, for example, can be exposed to carcinogens and the knockout tissues observed to determine if tumor formation occurs in the absence of telomerase. Therefore, the present invention provides a method to determine the relationship between telomere shortening, telomerase activity and tumor formation.

As an alternative, an exogenous gene encoding a telomerase component can be combined with an inducible promoter and integrated into the genome of a knockout organism of the present invention so that telomerase expression can be turned on or off at certain times or in particular tissues of the transgenic organism. Also, a "conditional knockout" can be generated (Example 8) if a knockout transgenic organism has a significantly reduced viability, making it difficult to breed, or if the absence of telomerase in all tissues results in additional phenotypes that complicate studies of the effects of

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telomerase deletion. In one embodiment, a mouse is produced that is heterozygous for the mTR knockout while the endogenous mTR gene is flanked by a recombination site on either side of the gene. When the mouse is bred to a mouse with a gene encoding recombinase, most of the cells of the F1 mouse have one copy of the recombinase. Thus, in selected tissues, telomerase expression is completely knocked out.

The alternative transgenic nonhuman organisms described above are useful if a knockout embryo of an animal species other than a mouse cannot survive. Telomerase transcription in the knockout can be turned off at any stage of development to ascertain when and where telomerase is essential, especially in growth and aging processes, or in tumor formation. Alternatively, telomerase transcription in an adult animal can be turned on or off in selected tissues so that the effects of a drug can be determined with and without telomerase activity.

Modifications of organisms through transgenic procedures can produce telomerase alterations of various types, including insertions, deletions, substitutions, or additions of nucleic acids or amino acids, or any combination of the preceding. A specific example of such a modification is the inactivation of the telomerase RNA component gene by site-specific integration of a nucleotide sequence that replaces the endogenous gene encoding the RNA telomerase component of the mouse, as described in the Exemplification. Using this technique to knock out a gene by gene targeting avoids problems associated with the use of antisense RNA to disrupt functional expression of a gene product. As described in Examples 2 and 3, a selectable marker gene, flanked by DNA sequences isogenic to the sequences at the 5' and 3' most ends of the gene segment to be replaced, is inserted into the exogenous DNA construct so that homologous recombination between the exogenous

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construct and the endogenous target DNA results in insertion of the selectable marker gene into a coding region or essential regulatory element of the target gene. The term "selectable marker gene" refers to a nucleic acid
5 sequence whose expression allows for selection of targeted cells that have stably incorporated the exogenous DNA, making it possible to screen the targeted cells or derivatives of these cells for heterozygosity.

Examples of genes encoding selectable markers include,
10 but are not limited to: genes conferring resistance to compounds such as antibiotics, genes conferring the ability to grow on selected substrates, and genes encoding proteins that produce detectable signals, such as dye staining or luminescence. A wide variety of such markers are known and
15 available, including, for example, antibiotic resistance genes such as the neomycin resistance gene (NEO), Southern, P. and Berg, P. (1982) *J. Mol. Appl. Genet.* 1:327-341 (1982); and the hygromycin resistance gene (HYG) Kaster, K., et al. (1983) *Nucleic Acids Res.* 11:6895-6911, and Te
20 Riele, H., et al. (1990) *Nature* 348:649-651. Other selectable markers for use in organism cells, and plasmids carrying a variety of genes encoding selectable markers, are described in Sambrook, J., et al. *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold
25 Spring Harbor, N.Y. (1989).

The invention also provides embryonic stem (ES) cells and embryonic stem cell lines in which an endogenous telomerase component gene has been knocked out (deleted or otherwise disabled) by the methods described herein. A
30 further subject of the invention are ES cells and ES cell lines in which a telomerase component gene has been replaced with a different gene. The replacement gene can encode a chimeric product; for example, a gene encoding a mouse/human telomerase RNA component wherein the human
35 template sequence replaces the mouse template sequence in a

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mouse telomerase RNA component gene. Such a construct is useful to determine if human telomerase can be produced and are capable of elongating the telomeres of mouse chromosomes. A replacement gene can also encode a complete or functional portion of a telomerase RNA or protein component sequence from another organism species. Several examples of genes encoding telomerase RNA or protein components are shown in Figures 5, 6, 7A-7B, 8, and 10. Active telomerase comprising chimeric combinations of RNA and protein components of different species can provide valuable information concerning phylogenetic origins and can also provide therapeutic models for recombinant molecules that can be used in gene therapy. It could further be determined if a construct for replacement of the RNA component gene which retains the conserved nucleotides of the genes encoding telomerase RNA component of different species, as shown in Figure 7A-7B, can produce active telomerase in any organism.

Transgenic organisms in which the gene encoding telomerase can be turned on or off in cells or tissues are useful for studying aging and control of tumor growth, as well as many other growth processes. For this purpose, chimeric sequences which comprise an inducible promoter coupled to a telomerase component gene can be used to increase or decrease telomerase activity in particular cells and tissues at preferred times. For example, the tetracycline-responsive cytomegalovirus promoter effectively allows differential control of the activity of genes in mammalian cells. Gossen, M. and Bujard, H. (1992) *Proc. Natl. Acad. Sci. USA* 89:5547-5551. Cells and tissues comprising telomerase component genes under such controls are useful in the study of cellular senescence and immortalization.

The capability to maintain chromosomal replication (i.e., cell division) has many therapeutic possibilities,

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among which is gene therapy. During gene therapy, cells are normally extracted from individuals, the genome of the cell is altered to correct a genetic defect or produce a required protein (such as erythropoietin), and the altered cells returned to the individual. If immortalized cell lines are not used, the extracted cells are limited in their number of cell divisions so that small numbers of therapeutic cells are produced. Instead of using immortalized cell lines, which raises the possibility of tumor induction, blood or tissue cells of an individual can be genetically modified to incorporate genes encoding telomerase as well as nucleic acids encoding a therapeutic protein or proteins. The encoded telomerase can lengthen the number of cell divisions, thus lengthening the life span of the modified cells prior to therapy or after introduction into an individual. Larger numbers of therapeutic cells could be produced for delivery to the individual. (The individual from which the cells were obtained or another individual.) The prophylactic and therapeutic possibilities are not limited, and include any application wherein the control of cell division, is beneficial to the organism.

A replacement sequence for an endogenous telomerase gene, e.g., the sequence diagrammed in Figure 1B, can encode a marker, such as a neomycin resistance gene. The marker gene can be coupled to a promoter which is constitutive or inducible. It is expected that the expression of the marker will be used to detect cells wherein the marker DNA sequence has replaced an endogenous telomerase component sequence and homologous recombination has occurred.

This invention also includes constructs, particularly DNA constructs, useful for producing the transgenic nonhuman organisms, such as the transgenic mice, described herein. The DNA comprising the knockout or replacement

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construct will usually include one or more exon(s) and/or intron(s), or regions thereof of a gene encoding a telomerase component, and/or a promoter region. Any DNA that is the functional equivalent of a telomerase component
5 can be used. The functional equivalent of a telomerase component gene is a gene encoding a molecule that is capable of combining with other endogenous or exogenous telomerase components to form an active telomerase enzyme. Other DNA can comprise promoters, sequences which encode
10 markers, and modified or synthetic gene sequences.

Generally, the DNA construct will be at least about 1 kilobase (kb) in length and preferably 3-15 kb in length, thereby including sufficient complementary sequence for recombination when the exogenous construct is introduced
15 into the genomic DNA of the targeted cell. Larger constructs may be required if the replacement is comprised of several genes and promoters or regulatory sequences. The preferred genes to be altered are any or all of the genes encoding the RNA component and the protein
20 component(s) of telomerase.

There may be one or more protein components comprising the telomerase enzyme, depending on the species of cell targeted. The *Tetrahymena* telomerase ribonucleoprotein, for example, contains a 95 kb protein (p95), an 80 kb
25 protein (p80) (Figures 9 and 11, respectively), and a 159 nucleotide RNA component. The RNA component contains a short internal sequence which serves as a template for synthesis of the G-rich strand of a telomeric repeat. The two protein components have different nucleic acid binding
30 properties: p95 binds specifically to telomeric primer DNA, whereas p80 binds most specifically to the telomerase RNA.

Both the RNA component and the protein component of telomerase are essential for enzymatic activity.
35 Therefore, it is only necessary to ensure that one of the

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telomerase components, either the RNA component or a protein component, is not transcribed or that the product of the gene is not functional to diminish or suppress the activity of telomerase. As used herein, the term

5 "nonfunctional" refers to the inability of the expressed component to combine with any of the other telomerase components to form an enzyme capable of adding telomeric repeats to chromosome ends or to the inability to perform its specific enzymatic role once combined.

10 The DNA sequence used in producing the knockout construct is digested with a particular restriction enzyme selected to cut at a location such that a new DNA sequence encoding a marker gene can be integrated in a position within this DNA sequence and transcription of the
15 endogenous gene will be prevented after insertion of the knockout construct into the chromosome. Those of skill in the art will recognize the various factors that will affect the selection of useful restriction sites for this purpose. The marker gene to be inserted will normally have a polyA
20 addition site attached to its 3' end. The marker can be operably linked to its own promoter or to another strong promoter from any source that is active or can easily be activated in the cell into which it is integrated. Alternatively, the marker gene can be transcribed using the
25 promoter of the gene that is suppressed.

By flanking the exogenous gene of the construct with sequences substantially isogenic with the target DNA in the host cell, it is possible to introduce the gene in a site-specific fashion at the targeted location. Using this
30 approach, a gene from any source (e.g., bacterial, fungal, plant, animal) can be introduced into the organism host cell to impart new telomerase characteristics to the cell, replace a telomerase component, or otherwise alter the cell to produce desired telomerase products which can then be
35 used to assay *in vivo* for the effects of new drugs.

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Isolated cells and tissues from the organism or from its descendants can also be used for assays *in vitro*.

An example of a knockout construct of the present invention is shown in Figures 1B and 4. The plasmid pPNT-
5 mTRA comprises the selectable marker gene for neomycin resistance inserted in a position where it will replace the endogenous gene which encodes the RNA component of mouse telomerase. See Figure 1A. The targeted endogenous
10 sequence to be deleted comprises a 3.9 kb segment which includes the gene encoding the mouse telomerase RNA component (mTR), and is located between a 3.3 kb *Xba*I fragment at the 5' end and a 4.0 kb *Xho*I fragment at the 3' end of the endogenous sequence.

The replacement sequence is ligated into the genomic
15 DNA sequence after the genomic DNA sequence has been digested with the appropriate restriction enzymes. To make the ends of the fragments compatible for ligation, the ends can be blunted, for example by Klenow fragment, or all fragments can be cut with enzymes that generate compatible
20 ends. Methods for carrying out these procedures are well known to those skilled in the art and can be found in Sambrook *et al.*, *supra*. The ligated construct can be inserted directly into ES cells or it can be incorporated into a suitable vector for amplification prior to
25 insertion.

Transgenic animals from any species of rodent, including without limitation, rabbits, rats, hamsters, and mice, can be produced, as can other nonhuman transgenic organisms, such as dog, cat, pig, sheep, cow and primates.
30 In most cases, the ES cells used to produce the transgenic animal will be of the same species as the transgenic animal to be generated. Thus, for example, mouse embryonic stem cells will usually be used for the generation of knockout mice. Transgenic animals can be prepared using methods
35 known to skilled artisans. See, for example, Hogan, *et al.*

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(eds.), *Manipulating the Mouse Embryo: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1986).

The ES cells can be selected for their ability to
5 integrate into and become part of the germ line of a developing embryo so as to result in germline transmission of the inserted DNA construct. Thus, any ES cell capable of such integration is suitable for use herein.

Methods of insertion of DNA vectors into ES cells
10 include, but are not limited to, electroporation and microinjection. The insertion of DNA constructs into ES cells by electroporation is described in Example 3 of the Exemplification.

ES cells can be screened for the inserted construct by
15 a variety of methods, which can be used alone or in combination. If a marker sequence has been incorporated, the appropriate conditions and procedures are applied to identify the marker product in the cells as an indication of the presence of the construct. For example, where the
20 marker is an antibiotic resistance gene, such as the neomycin resistance gene, the cells are contacted with a concentration of the antibiotic (neomycin) which is lethal to cells which do not express a functional neomycin resistance gene. Those cells that survive are presumed to
25 have integrated the construct into their genome because they are resistant to neomycin, whereas the wildtype cells are not. Or, the marker gene can encode an enzyme whose activity is detected by contacting the cells with the appropriate substrate for the encoded enzyme, then assaying
30 for enzyme activity (or product).

Alternatively, or in addition, a Southern blot of the genomic DNA of the ES cells can be probed with a DNA
sequence which hybridizes to the marker sequence. Probes
such as the 1.1 kb and 1.2 kb probes shown in Figure 1A and
35 2, can be used to distinguish the genome of ES cells or

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tissues of transgenic organisms which are either unchanged or in which the inserted construct has randomly integrated into the genome from those in which the knockout construct has integrated into targeted site (mTR) of the genome.

- 5 These probes are especially useful to determine if the germline cells of the transgenic organism include a knockout construct for the telomerase RNA component.

Figure 1A illustrates a 1.1 kb probe at the 5' flanking end of the wildtype chromosome and a 1.2 kb probe
10 at the 3' flanking end of the same chromosome, either of which can be used to determine if the knockout construct of pPNT-mTRA has replaced the endogenous mouse telomerase RNA component gene through homologous recombination. The 5' flanking probe is located between *SacI* (*Sc*) and *XbaI*; the
15 3' flanking probe is located between *XhoI* and *SacI*. Following digestion with enzyme *SacI*, the fragment lengths of DNA isolated with these probes will be shorter after excision from the knockout construct due to the insertion of a unique *SacI* (*Sc*^{*}) restriction site engineered into the
20 *XbaI* and *XhoI* genomic fragments which are incorporated into the knockout construct (see Example 2). As shown in Figure 1A, the wildtype gene will produce a 7.0 kb fragment including the 5' probe and a 6.5 kb fragment with the 3' probe. If the NEO gene has replaced the mouse telomerase
25 gene, a 4.4 kb fragment (5' probe) or a 5.2 kb fragment (3' probe) will be produced. Therefore, if the 5' most probe is used for detection, wildtype cells will produce only a 7.0 kb band; a heterozygous cell will produce 7.0 kb and 4.4 kb bands, and those cells homozygous for the knockout
30 construct (homozygous null) will produce a 4.4 kb band only. In a similar manner, the bands detected with the 3' most probe will be 6.5 kb (wildtype cells), 6.5 kb and 5.2 kb (heterozygous cells), or 5.2 kb (homozygous null cells). Those of skill in the art will recognize that other probes
35 can be isolated and used with these methods to determine if

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replacement of a telomerase component gene has occurred in cells and tissues of a transgenic organism.

The ES cells carrying a knockout or altered construct are introduced into embryos using known methods. For
5 example, they can be microinjected into eggs according to known protocols, such as those described in Example 5. Other methods for production of transgenic rodents are set forth in Hogan, et al. (eds.), *Manipulating the Mouse Embryo: A Laboratory Manual*, Cold Spring Harbor Laboratory
10 Press, Cold Spring Harbor, N.Y. (1986).

The developing ES cell carrying the construct is injected into a blastocyst and implanted into a pseudopregnant female of an appropriate strain and allowed to develop into offspring. As an option, the blastocyst
15 can be fertilized and incubated *in vitro* prior to introduction into the female.

Following implantation, gestation, and birth, the nonhuman organism which develops from the embryo is a transgenic nonhuman organism, preferably one in which the
20 germline cells contain the altered construct. These founder organisms, which are likely to be heterozygous for the altered construct, can be bred to produce homozygotes. See Example 6. Heterozygotes, as well as being useful to generate knockout organisms, can be altered to produce
25 further genetic modifications affecting telomerase activity resulting from the expression of an endogenous telomerase component gene. Conditional knockout organisms as described in Example 7 represent one application of a modification.

30 The transgenic organisms and cells derived from such organisms have a variety of uses, which depend on the telomerase component expressed and their integrated constructs. Such organisms can be used to screen for drugs or a therapeutic regimen useful for prophylactic or
35 therapeutic treatment of diseases such as cancers and for

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the regeneration of cells and tissues that do not normally divide infinitely.

More specifically, this invention provides a method of identifying an agent that is active in stimulating

5 telomerase activity, which comprises:

- a) administering an agent to a transgenic nonhuman organism, wherein the organism does not express telomerase activity or has diminished telomerase activity but has the capability to produce
10 telomerase; and
- b) assessing the effect of the agent on the telomerase activity,

wherein if the agent causes an increase in telomerase activity, the agent is active in stimulating telomerase
15 activity.

This invention further provides a method of identifying an agent that is active in stimulating telomerase activity, which comprises:

- a) administering an agent to a sample of transgenic
20 cells or tissue, wherein the sample does not express telomerase activity or has diminished telomerase activity but is capable of producing telomerase activity; and
- b) assessing the effect of the agent on the
25 telomerase activity,

wherein if the agent causes an increase in telomerase activity, the agent is active in stimulating telomerase activity.

Additionally, this invention provides a method of
30 identifying an agent that is active in inhibiting telomerase activity, which comprises:

- a) administering an agent to a transgenic nonhuman organism, wherein the organism expresses telomerase activity; and
- 35 b) assessing the effect of the agent on the

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telomerase activity,
wherein if the agent causes a decrease in telomerase
activity, the agent is active in inhibiting telomerase
activity.

5 The method can also be used with samples of transgenic
cells or tissue which express telomerase and are derived
from transgenic nonhuman organisms to determine if the
agent inhibits telomerase activity.

Just as the organisms of this invention can be used as
10 models to study telomerase activity, they can also provide
a system in which a material suspected of being a
carcinogen can be tested by exposing the organism to the
material and determining neoplastic growth as an indicator
of carcinogenicity. Transgenic nonhuman organisms, in
15 particular, are very useful to develop effective therapies
or regimens for combatting diseases or conditions and to
ascertain the overall specific effects of a drug on
telomerase activity in a living system.

The role of telomerase in the development and growth
20 of tumors can be determined by comparing the effects of
carcinogens and other tumor-inducing mechanisms in tissues
wherein telomerase activity is turned on or off. See,
e.g., Examples 7 and 8. Telomerase activity in tumor cells
maintains the severely-shortened telomeres of these cells.
25 The absence of telomerase in tumor-forming cells could
retard and even stop the growth of tumors if the dividing
tumor cells lose their telomeres completely due to lack of
telomerase activity.

Another important feature of the present invention is
30 that it provides, for the first time, an organism wherein
the side effects of drugs affecting telomerase activity can
be ascertained and distinguished from the effects of
diminished or enhanced telomerase activity. By "side
effects" it is meant those effects of a drug which are not
35 attributable to the alteration in telomerase activity.

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Further, the side effects of such drugs as they affect the descendants of treated organisms can be determined. In particular, the toxicity of telomerase activity inhibitors can be tested *in vivo* using mammalian systems such as the transgenic mice described herein. For example, genes encoding human telomerase can be used to replace endogenous telomerase genes in mice, and the effects of telomerase inhibitors can be studied in a mammalian system over several generations so that any short-term or long-term side effects of the inhibitors can be documented along with the specific effects of alterations in telomerase activity. In addition, screening of these drugs for their effects on particular transgenic cells and tissues can be done *in vivo* or *in vitro*.

More specifically, this invention provides a method of identifying side effects of an agent that modifies telomerase activity in an organism, which comprises:

- a) administering the agent to a transgenic nonhuman organism which is incapable of expressing telomerase; and
- b) assessing the effects of the agent on the transgenic nonhuman organism,

wherein if the agent causes one or more effects on the organism, it is identified as an agent that causes one or more side effects.

In knockout organisms or tissues, shutting down the production of telomerase may result in the inability of cells to immortalize, thus, making tumor formation impossible. In addition to using knockout organisms for demonstration of these effects, cells or tissues comprising the knockout construct can be transplanted onto nude mice which have a propensity to form tumors. The absence or reduced incidence of tumors at the transplantation sites would show that telomerase is required for tumor formation.

Alternatively, conditional knockout organisms or those

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in which a telomerase component is under the control of an inducible promoter, as described *supra*, can be used to induce suppression of telomerase production either before or after tumor formation. It could then be determined if tumor formation can occur in the absence of telomerase and if suppression of telomerase causes cell division to cease in tumors.

In particular, mouse model systems for tumor formation have generated a wealth of information about cancer progression. Palmiter, R.D. and Brinster, R.L. (1985) *Cell* 41:343-345; Cory, S. and Adams, J.M. (1988) *Ann. Rev. Immunol.* 6:25-48; Hanahan, D., et al. (1989) *Science* 246:1265-75. The expression of oncogenes in transgenic mice provided definitive evidence that oncogenes cause tumors in mammals. Further, the finding that not all cells that express these oncogenes form tumors supported the multiple hit model for tumor progression. Brinster, R.L., et al. (1984) *Cell* 37:367-379; Adams, J.M., et al. (1985) *Nature* 318:533-538; Hanahan, D. (1985) *Nature* 315:115-122; Cory and Adams, *supra*. Mouse models for tumor suppressor genes have been developed by creating homozygous germline deletions in mice. Donehower, L.A., et al. (1992) *Nature* 356:215-221; Jacks, T., et al. (1992) *Nature* 359:295-300; Jacks, T., et al. (1994) *Curr. Biol.* 4:1-7; Jacks, T., et al. (1994) *Nature Genet.* 7:353-361. Mice that are homozygous null for a telomerase component, such as the mTR (-/-) mice, or heterozygous mTR (+/-) mice can be crossed to both oncogene expressing mice and mice deleted for tumor suppressor genes, and the mTR (-/-) oncogene-expressing offspring examined to determine if the absence of telomerase results in a lower rate of tumor formation, smaller tumor size or a lower frequency of metastasis. Further, a recombination deficient mouse can be used to generate a mTR (-/-) mouse into this background to look at the effects of recombination telomerase bypass pathways.

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See Example 8.

An organism or tissue system in which telomerase activity is under the control of an inducible promoter, can also be used to study senescence. Most somatic cells lose
5 segments of their telomeres as they divide in culture; whereas, telomeres are maintained in germline cells. Greider, C. and Blackburn, E.H. (1996) *Scientific American* 274(2):92-97. Stimulation of telomerase activity in
10 somatic cells of an aging transgenic nonhuman organism could answer many questions about the role of telomerase during periods of cellular senescence, especially the effects of telomerase in atherosclerosis or the decline in immunity individuals experience as they age.

Those skilled in the art will also recognize the value
15 of the organisms, cells and tissues of this invention as a tool for understanding the growth and division of cells, and for production of cells and tissues that, in their wildtype form, are difficult to study *in vitro* because they undergo a small number of cell divisions.

20

EXEMPLIFICATION

Example 1Map of mTR Genomic Locus

A 15 kb lambda genomic clone containing the mouse telomerase RNA gene was subjected to restriction enzyme
25 digestion and various resultant fragments were subcloned into a Bluescript vector for further analysis. To map the different genomic fragments, Southern blots were probed with a 2 kb genomic fragment containing the mTR gene. Restriction enzyme sites are shown for the following
30 enzymes; *SacI*, *XhoI*, *EcoRI*, *XbaI*, *EcoRV*, *BamHI*, and *PstI* (Figure 2).

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Example 2Construction of the Targeting Vector

To generate a mTR genomic deletion construct, site-directed mutagenesis was used to introduce two new unique
5 SacI restriction sites (SacI* or Sc*) into the 3.3 kb XbaI
and 4.0 kb XhoI genomic fragments (Figure 1A). These
unique sites were engineered so that the correct homologous
recombination events in ES cells could later be identified
by digestion of genomic DNA with SacI. See Figures 1A and
10 4. Using a 1.2 kb probe (mTR probe) to the right of the
mTR gene following digestion with SacI yields a 6.5 kb
wildtype band or a 5.2 kb band from the correctly targeted
deletion allele. The probe was labeled by random primer
extension using Klenow enzyme, ³²P-dGTP (3000 Ci/mmol),
15 ³²P-dATP (3000 Ci/mmol) and cold dCTP and dTTP.

To introduce the SacI* sites, both the XbaI and XhoI
genomic fragments were cloned into Bluescript SK- and KS+,
respectively (Figures 3 and 4), and the orientation of the
clones was determined by restriction enzyme digests.
20 Approximately 250 nt of the 3' end of XbaI fragment and the
5' end of the XhoI fragment were sequenced and
oligonucleotides were designed to introduce the SacI* sites
by site-directed mutagenesis.

The engineered SacI* restriction sites were
25 introduced into a subcloned XbaI 4.0 kb fragment located 5'
of the transcribed region and a subcloned 3.3 kb XhoI
fragment located 3' of the transcribed region. The
creation of these restriction sites was confirmed by
sequencing and restriction enzyme digests. After the
30 mutagenized fragments were sequenced, they were cloned into
the targeting vector pPNT (Tyulewicz, V.L., et al. (1991)
Cell 65:1153-1163) to generate the plasmid pPNT-mTRΔ (See
Figures 1B and 4). This vector contains a Neomycin
resistance gene for positive selection of resistant clones
35 and the HSV-tk gene (Thomas, K.R. and Cappechi, M.R. (1987)

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Cell 51:503-512) for counter selection in gancyclovir of incorrectly integrated constructs.

The arrangement of the two mTR genomic fragments in the targeting construct pPNT-mTRA is such that when
5 homologous recombination occurs after electroporation of mouse ES cells, the mTR gene is replaced by the Neomycin resistance gene (NEO).

Example 3

Electroporation of pPNT-mTRA into WW6 ES Cells

10 WW6 ES cells (E. Ioffe, et al. (1995) *Proc. Natl. Acad. Sci.* 92:7357-61) were thawed and grown in ES medium with Leukemia Inhibitory Factor (LIF). The plasmid pPNT-mTRA was linearized with the restriction enzyme NotI, and the DNA was electroporated into WW6 cells by standard
15 protocols. Ramirez-Solis, R., et al. (1993) *Meth. Enzymol.* 225:855-929. Cells were selected in G418 (100 µg/ml) and gancyclovir (2 µM), and positive clones were picked after 9-10 days growth into duplicate 48 well microtitre plates; one for DNA analysis, the other for both freezing at -80°
20 C. and potential blastocyst injection. Cells in DNA plates were grown to near confluency, harvested in Proteinase K solution (0.1% SDS, 0.5 mg/ml of Proteinase K), and analyzed for homologous recombination.

Example 4

25 Southern Analysis of Doubly-Resistant ES Cell Clones

Genomic DNA was prepared from over 400
G418/gancyclovir resistant cells grown in one well of a 48 well-plate after digestion with Proteinase K solution for one hour at 37° C. followed by phenol extraction and
30 isopropanol precipitation. Approximately 20 µg of DNA was digested overnight with 40 units of SacI, in the presence of RNase A. After digestion, samples were loaded on a 0.8% agarose/1X TBE gel containing Ethidium bromide and

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subjected to electrophoresis for 24 hr at constant voltage (30 volts). For Southern analysis, the gels were denatured and neutralized following standard protocols and transferred to a nylon filter (Hybond N+ Amersham). After transfer, filters were washed in 2X SSC and the DNA was UV-crosslinked to the membrane using a Stratalinker. Filters were pre-hybridized in high stringency solution (1% bovine serum albumin, 200 mM sodium phosphate, 15% formamide, 1 mM EDTA and 7% sodium dodecyl sulfate) at 65° C. for two hours. Hybridization with an mTR probe (described below) was carried out overnight in the same solution. After hybridization the blots were washed in 0.2X SSC and 0.1% SDS at 65° C. and exposed to autoradiographic film.

Example 5

15 Generation of Chimeric and Heterozygous Mice Deleted for mTR

Of the 400 Neomycin/gancyclovir resistant clones initially screened, four were found by Southern blot hybridization to contain one correctly targeted mTR locus that was deleted for the mTR coding region. These four clones were designated Tel-1, Tel-2, Tel-3 and Tel-4. Each clone was thawed from the frozen 48 well-plate and grown in culture before injection into C57BL/6J blastocysts. Three of the clones (Tel-1, Tel-2, and Tel-3) were independently injected and the blastocysts were implanted into pseudo-pregnant mice. (See Table 1 for numbers of injected mice). The chimeric mice which were born from these mothers were identified by their mosaic coat color (resulting from the agouti contribution of the ES cells and the black contribution of the blastocysts). The mice generated from the Tel-1 microinjections had over 90% ES contribution from the injected WW6 cells, based on the percent of agouti versus black coat color.

These chimeric mice were then mated to C57BL/6J to

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test for germline transmission of the knocked out allele. The number and sex of chimeric and heterozygous mice from each injection are shown in Table 1. Tail DNA from the progeny of this cross was analyzed by the Southern blot
5 procedure described above using the unique 1.2 kb genomic mTR probe. Heterozygous mTR (+/-) mice having one wild type allele and one knockout allele were considered to have germline transmission for the targeted mTR (+/-) WW6 ES cells.

10

Example 6Generation of Mice Homozygous null for mTR

Homozygous knockout mice can be generated by three methods:

a) Initially a male chimeric mouse which has
15 previously exhibited germline transmission is mated to a heterozygous female mTR knockout mouse. If telomerase null mice are viable, up to 25% of the progeny born from this cross are expected to be mTR (-/-).

b) Homozygous mTR mice are also generated by
20 directly crossing sibling male and female heterozygotes (mTR (+/-) sibling mating). In this cross, 25% of the progeny are expected to be mTR (-/-).

c) ES cells homozygous null for mTR can be generated from intrachromosomal homologous recombination at increased
25 concentration of G418 (higher than 1 mg/ml). These homozygous null ES cells are injected into C57BL/6J blastocysts and transferred to the uteri of pseudopregnant females. Resultant chimeric mice contain varying ES-
contribution to somatic tissues. An inert molecular tag
30 permits an assessment as to whether cells are derived from ES or host blastocysts.

The method described in b) was used to produce homozygous null mice. See Table 1.

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Table 1
Production of Telomerase-Deficient Mice

Telomerase (+/-) ES Cell Clone	Number of Blastocysts Injected	Number of Chimeras (Chimerism)	Number of Germline Chimeras	Number of (+/-) Animals	Offspring from (+/-) x (+/-) (-/-)
Tel-1	124	5♂ + 6♀ (60-95%)	4♂ + 1♀	12♂ + 12♀ DOB 9/29 -10/18/95	A 2 5 0 B 0 3 3 C 2 3 1 D 1 3 0 E 3 5 0 F 0 3 1 G 2 3 0 H* 3 5 1
Tel-2	53	6♂ + 4♀ (40-80%)	mated		
Tel-3	134	9♂ + 3♀ (60-100%)	mated		
Tel-4	not injected				

* Embryos were taken prior to birth and used for generation of fibroblast cultures.

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A total of 8 transfections were performed with approximately 2×10^7 or 4×10^7 WW6 ES cells per transfection. Six transfections with an average enrichment of 4.50 provided 158 clones which were analyzed by Southern blot, demonstrating one heterozygous null (+/-) cell line (Tel-1). The average enrichment for two other transfections (where 4×10^7 cells were electroporated), was 5.75. Three (+/-) ES clones (Tel-2, Tel-3 and Tel-4) were isolated among 280 clones screened. In summary, the homologous recombination event of telomerase occurred at 0.17% (four in 2321 clones) in WW6 ES cells. The low frequency of homologous recombination made it difficult to obtain mutant ES clones. Therefore, efficient subcloning and screening strategies were developed which permitted a more rapid and extensive analysis of candidate mutant clones. These procedures were crucial to the success of this work. Moreover, a unique ES cell line, WW6, was important to the success of the gene targeting experiments. The mixed genetic composition of WW6 provides enhanced capacity to contribute to competent germ cells. Virtually all of the mutant ES cell clones that have been generated with this cell line have passed the mutant allele through the germline.

One heterozygous null (+/-) clone (Tel-1) has transmitted through the germline, producing animals that are heterozygous null (+/-) for telomerase. Heterozygous null animals were intercrossed to examine the viability of homozygous null animals. Because WW6 ES cell line has a mixed genetic background (75% 129/sv, 20% C57Bl/6J, and 5% SJL), some of the (+/-) animals were crossed with C57Bl/6J mice to produce the inbred strain of C57Bl/6J.

To generate homozygous knockout mice, heterozygous mTR (+/-) mice were crossed and the progeny examined by Southern blotting of tail DNA. Of 46 progeny examined from

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the first eight litters, three homozygous null mTR (-/-) pups were found (Table 1). The initial screening was done using SacI cut genomic DNA looking for a 5.2 kb band diagnostic of the deleted allele in place of the wildtype 6.5 kb band (Figure 1A). Several control procedures confirmed that the mice were null for mTR. Genomic DNA was cut with EcoRI and probed with the coding region for mTR. The wildtype and heterozygous pup DNA produced a 5.0 kb band; whereas, this band was absent in the deleted mTR (-/-) DNA. PCR was also carried out, using primers in mTR and just inside the XhoI site to the right of the gene. A correctly-sized band of 1.3 kb was generated with the wildtype and heterozygous DNA but not with the homozygous null DNA.

Of ten pups born in the first two litters of the mTR (+/-) intercross, none were homozygous null (2 were (+/+) and 8 were (+/-)). However, the subsequent litters produced 24 homozygous null pups out of 93 born (31 homozygous normal and 62 heterozygous), indicating that animals without a gene encoding a telomerase RNA component can survive to birth. The mTR (-/-) pups are similar to their littermates in size and appearance. Both cells and tissues derived from homozygous null mice lack telomerase activity.

25

Example 7

Generation of Conditional mTR Deletions

Several techniques for producing conditional knockout mice are known in tissue culture (Sauer, B. and Henderson, N. (1988) *Proc. Natl. Acad. Sci.* 85:5166-5170) and can be modified to produce mice with conditional deletions of either mTR or a telomerase protein component which are specific to certain tissues. In one procedure, the genomic region to be deleted is flanked by Lox P recombination sites. Lox P sites are DNA sequences recognized by the

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bacteriophage P1 Cre recombinase. Cre-mediated recombination at Lox P sites generates a site-specific deletion which leaves only one copy of Lox P in the genome. Sauer and Henderson, *supra*. This method has been highly successful. Studies assaying the deletion of polymerase β have reported up to 95% deletion in tissues such as liver, and as much as 60% deletion, even in non-dividing tissues such as heart. Kühn, R., et al. (1995) *Science* 269:1427-1429. Thus a mouse can be generated wherein one allele of mTR is deleted, and the second allele is flanked by Lox P sites. Knockout mTR (-/-) tissues are then generated by the expression of Cre in specific tissues.

Although mice which ubiquitously express Cre can be generated, tissue specific expression is more useful. Several techniques can be used to produce differential expression of mTR or another telomerase component. Cre can be placed behind a tissue specific promoter to generate specific deletion in a given tissue. Gu, H., et al. (1994) *Science* 265:103-106. Some promoters produce low level recombination and occasional leaky expression in unrelated tissues using this technique. To avoid these problems, Cre can be placed behind a tissue specific promoter and fused to a ligand-binding domain such as the domain from the estrogen receptor. Picard, D. (1993) *Trends Cell Biol.* 3:278-280; Logie, C. and Stewart, F. (1995) *Proc. Natl. Acad. Sci.* 92:5940-5944. Fusion to ligand-binding domains results in an inactive protein until the appropriate ligand is added to release the inhibitory effect of the ligand-binding domain. Picard, D., et al. (1988) *Cell* 54:1073-1080. Tissue specific expression can then be induced by feeding the mice the appropriate receptor ligand. Although the estrogen receptor has been used for many studies *in vitro*, the presence of estrogen *in vivo* can activate this receptor. Thus mutant receptors can be used which respond to artificial, non-physiological drugs.

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Example 8Tumor Induction in Telomerase-deficient mice

Rates of tumor formation in four different mouse tumor progression models, RIP-Tag2 insulinomas, K14-HPV squamous cell carcinoma, E μ -Myc lymphomas and p53 null mice can be determined. Mice which are homozygous null (-/-), wildtype (+/+) and heterozygous (+/-) for mTR are crossed to transgenic mice with high rate of tumor formation.

a) Pancreas and skin tumor models.

10 Telomerase expression in two mouse tumor models (RIP-Tag2 and K14-HPV16) has been extensively characterized. RIP-Tag2 mice and K14-HPV16 mice are crossed with mice heterozygous for mTR (+/-). Because genetic background affects the rate of tumor induction in mice, the mTR mice
15 that are in a mixed background of 129 and C57B1/6J are backcrossed to the appropriate strain to produce a more homogeneous genotype prior to crossing with mouse tumor models.

For the study of RIP-Tag2 induced insulinomas, mTR
20 (+/-) mice are crossed into the C57B1/6 strain, after which mTR (+/-) females are crossed to RIP-Tag2 males. It is not possible to introduce the RIP-Tag2 oncogene from the female side because diabetes is induced by the oncogene expression. Thus, for this model, the cross is made in
25 only one direction. Pups from this cross are genotyped and mTR (+/-) males carrying the RIP-Tag2 construct identified. These mice are subsequently mated to mTR (+/-) females. Approximately 12.5% of the pups from this cross should be mTR (-/-) and carry RIP-Tag2. These mice can be identified
30 through Southern blots of tail DNA. Rates of tumor formation, size of tumors and number of metastases in litter mates that are mTR (+/+), (+/-) or (-/-) are then determined. If the numbers of the mice with the appropriate genotype are low in some litters, siblings from

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identical crosses can be used as controls.

Similar experiments can be carried out to examine the rate of tumor formation in the K14-HPV16 squamous cell carcinoma model mice. The mTR (+/-) mice are backcrossed to FVB/n mice (Arbeit, J.M., et al. (1994) *J. Virol.* 68:4358-4368). Both male and female mTR (+/-) progeny are then crossed to K14-HPV expressing mice to generate mice carrying both mTR (+/-) and the K14-HPV transgene. Two heterozygotes carrying K14-HPV can then be crossed and Southern blots used to identify mTR (-/-) mice carrying the K14-HPV transgene.

In both the RIP-Tag2 and K14-HPV mouse models, telomerase activity appears to be present in the late stages of tumor progression, although telomerase RNA is upregulated early. Blasco, M., et al. (1996) *Nature Genetics* 12:200-204. However, not all tumors are telomerase positive, suggesting that telomerase is not absolutely required for progression to late stage tumors. Tumor progression is a stochastic process, not all cells which express the oncogenes become hyperproliferative and not all hyperplasias progress to tumor formation. If telomerase is required for those tumors where it is expressed, a comparison of mTR (-/-) to mTR (+/+) mice should show a reduced frequency of tumor formation in the mTR (-/-) mice.

If telomeres in mice are sufficiently long, telomere length may not be limiting for the number of divisions required to form a tumor. There is evidence that, unlike the tissue culture model where telomeres first shorten to a critical length, telomerase induction in tumors *in vivo* may not require critical telomere shortening. Mehle, C., et al. (1994) *Cancer Res.* 54:236-241. If no difference in tumor rates are found, mice with shorter telomeres can be generated and the experiment repeated using these mice.

Three different approaches can be taken to generate

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mice with shortened telomeres. First, if the mTR (-/-) mice are fertile, mice with shortened telomeres can be generated in a second generation mTR (-/-) mouse. Second, mTR (+/-) mice can be examined for telomere shortening. It is possible that half of the level of mTR is not sufficient for telomere maintenance and, consequently, telomere shortening results. If this occurs, the heterozygous mTR (+/-) mice can be used as a source of mice with pre-shortened telomeres. Finally, mice can be generated from mTR (-/-) ES cells that have been grown in culture for extended periods to allow telomere shortening. mTR (-/-) ES cells are generated by re-targeting the wildtype allele in the mTR (+/-) ES cells described *supra*. A vector similar to that shown in Figure 4 can be constructed which carries a hygromycin resistance gene in place of the mTR coding regions. ES cells can be electroporated and selected for growth in hygromycin. The doubly targeted cells are identified on Southern blots as described *supra*.

b) Lymphoma induction in mTR (-/-) mice.

Telomerase activity is present in lymphocytes in both humans and mice. In human B and T cell malignancies, telomerase activity is present at higher levels than in normal cells. Counter, C.M., et al. (1995) *Blood* 85:2315-2320; Broccoli, D., et al. (1995) *Proc. Natl. Acad. Sci.* 92:9082-9086. To determine if telomerase activity is required for induction of lymphomas, lymphoma induction in E μ -myc expressing mice (Jackson Laboratories, Bar Harbor, ME) with or without mTR can be compared. E μ -myc mice express the c-myc gene behind an immunoglobulin enhancer. These mice reproducibly develop pre-B cell lymphoma within a few months of birth. Adams, J.M., et al. (1985) *Nature* 318: 533-538. Crosses with mTR (+/-) mice can be used to generate E μ -myc expressing, mTR (+/-) heterozygotes. The rapidity with which lymphomas develop in this model can

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allow a tumor-suppressor phenotype to be identified in mTR (+/-) and (-/-) mice. Thus the generation of lymphomas in genetically identical litter mates that are mTR (+/+) or (+/-) can be compared. Finally, although tumors are
5 generated at early ages in E μ -myc expressing mice, it has been possible to generate males that will live to sexual maturity and to cross these males. Elson, A., et al. (1995) *Oncogene* 11:181-190. E μ -myc male, mTR (+/-) mice can be crossed to mTR (+/-) females to generate homozygous
10 mTR (-/-) mice expressing E μ -myc. The onset of lymphoma development and viability of the mice can then be monitored. If telomerase is essential for the long-term survival of B cells, lymphoma development may be retarded although immune dysfunction may still occur due to the loss
15 of B cells. The lymphoma cells from E μ -myc mice are easily cultured *in vitro*. Schmidt, E.V., et al. (1988) *Proc. Natl. Acad. Sci.* 85:6047-6051; Adams, J.M., et al. (1985) *Nature* 318:533-538. Pre-B cells will be cultured from the lymphomas in these animals and telomere length and the *in*
20 *vitro* life span of E μ -myc expressing mTR (+/+), (+/-) or (-/-) cells can be compared.

c) Effect of p53 deletion in mTR (-/-) mice.

The p53 gene is a tumor suppressor gene which is frequently mutated in a wide variety of human cancers.
25 Hollstein, M., et al. (1991) *Science* 253:49-53. Mice heterozygous for p53 develop tumors by around 16 months of age. Most of these tumors have suffered the loss of the wildtype allele of p53. Greater than 90% of mice with a complete (-/-) germline deletion of p53 develop tumors by
30 3-6 months of age. Donehower, L.A., et al. (1992) *Nature* 356:215-221; Jacks, T., et al. (1994) *Curr. Biol.* 4:1-7. To determine if the absence of telomerase decreases the tumor incidence or tumor size, mice doubly deficient for p53 and mTR can be generated. mTR (+/-) mice can be mated

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to p53 (+/-) mice and the tumor incidence and the tumor spectrum of the progeny examined for both litter mates and siblings with the following genotypes: mTR (+/+) p53 (+/+), mTR (+/+) p53 (+/-), mTR (+/+) p53 (-/-), mTR (+/-) p53 (+/-), mTR (+/-) p53 (-/-) and mTR (-/-) p53 (-/-). Since many human tumors are p53 negative, the affect of telomerase deletion on p53 null cells has important clinical implications. p53 null cells exhibit dramatic genomic instability. See, Lane, D.P. (1992) Nature 358:15-16. Telomere loss is also predicted to lead to genomic instability; the combination of telomerase and p53 loss may lead to cell death. If mTR (-/-) p53 (-/-) mice have reduced tumor incidence, telomerase inhibitors could be useful for treatment of p53-tumors in humans. Currently, p53 tumors are the most resistant tumors to chemotherapy.

d) mTR deletion in recombination deficient mice.

In the tumor induction models described above, telomerase negative tumors could survive due to telomere elongation via a recombination pathway. To date, no mice have been generated with a germline deficiency in recombination. In yeast, the Rad 52 recombination pathway is the best candidate for a potential mediator or telomere recombination because this pathway is essential for telomerase bypass in yeast. Lundblad, V. and Blackburn, E.H. (1993) Cell 73:347-360. Preliminary evidence suggests that deletion of the mouse Rad 52 gene is lethal. However, other genes in the same pathway could also allow recombinational bypass of telomerase deletion. The mouse homologues of Rad54 and Rad 51 have been cloned and Rad 54 (-/-) ES cells have been produced that are viable and recombination deficient. If either the Rad 54 or Rad 51 (-/-) mice are viable or if the heterozygotes show reduced recombination, these animals can be crossed to mTR (+/-) mice to generate recombination deficient mTR (-/-) mice.

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Fibroblasts from these cells can be transformed with Tag and ras, and tested for their ability to form tumors in nude mice as described supra. Ultimately, tumor induction in the transgenic models described above can be examined to
5 determine if recombination deficiency reduces the growth or formation of potential telomerase negative tumors.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many
10 equivalents to the specific embodiments of the invention described specifically herein. Such equivalents are intended to be encompassed in the scope of the following claims.

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Claims

We claim:

1. A nonhuman transgenic organism wherein at least one endogenous gene encoding a telomerase component is altered and telomerase activity is altered.
2. The organism of Claim 1 wherein the endogenous gene encodes all or part of the telomerase RNA component.
3. A cell or tissue obtained from the organism of Claim 1.
4. The organism of Claim 1 wherein the expression of telomerase activity is reduced as compared to a wildtype organism of the same species.
5. A cell or tissue of the organism of Claim 4.
6. A nonhuman transgenic organism wherein pPNT-mTRA is incorporated into the germline cells at the mTR locus.
7. A somatic or germline cell of a nonhuman animal comprising an exogenous telomerase gene sequence.
8. A germline cell according to Claim 7 which is an embryonic stem cell.
9. The organism of Claim 1 wherein the endogenous gene encoding a telomerase component is altered through insertion into the genome of the organism of a nucleic acid sequence comprising at least a portion of an isogenic coding sequence linked to a marker sequence.

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10. The marker sequence of Claim 9 that is a neomycin resistance gene.
11. A cell or tissue of the organism of Claim 9.
- 5 12. A nucleated cell of the organism of Claim 9 wherein the cell is selected from the group consisting of embryonic stem cells, embryonic fibroblasts, germline cells, somatic cells, white blood cells, and liver cells.
- 10 13. A nonhuman transgenic organism wherein an endogenous gene encoding a telomerase component, which is normally in the corresponding nonhuman wildtype organism, is not expressed.
- 15 14. The nonhuman transgenic organism of Claim 13 which is homozygous for an exogenous DNA sequence that replaces all or a part of an endogenous gene encoding the RNA component of telomerase.
- 15 15. The nonhuman transgenic organism of Claim 13 which contains no endogenous gene sequence capable of expressing functional telomerase.
- 20 16. The organism of Claim 13 which contains only one endogenous gene sequence per cell capable of expressing functional telomerase compared to the genome of a corresponding cell of a wildtype organism of the same species.

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17. A nucleic acid probe which can detect the length of a telomerase gene fragment, wherein the length of the fragment determines the capability of a telomerase gene sequence to encode a functional telomerase RNA component.
18. A DNA construct comprising DNA encoding exogenous DNA between the 3.3 kb XbaI fragment of the 5' end of the mouse telomerase RNA component gene and the 4.0 kb XhoI fragment of the 3' end of the mouse telomerase RNA component gene.
19. The construct of Claim 18 wherein the exogenous DNA is a marker gene sequence.
20. The construct of Claim 19 wherein the marker sequence is a neomycin resistance gene.
21. The construct of Claim 18 further comprising a promoter.
22. The construct of Claim 21 wherein the promoter is an inducible promoter.
23. The construct of Claim 22 wherein the inducible promoter is a tetracycline-responsive cytomegalovirus promoter.
24. A mouse embryonic stem cell containing the DNA construct of Claim 18.
25. A plasmid comprising pPNT-mTRA or its functional equivalent.

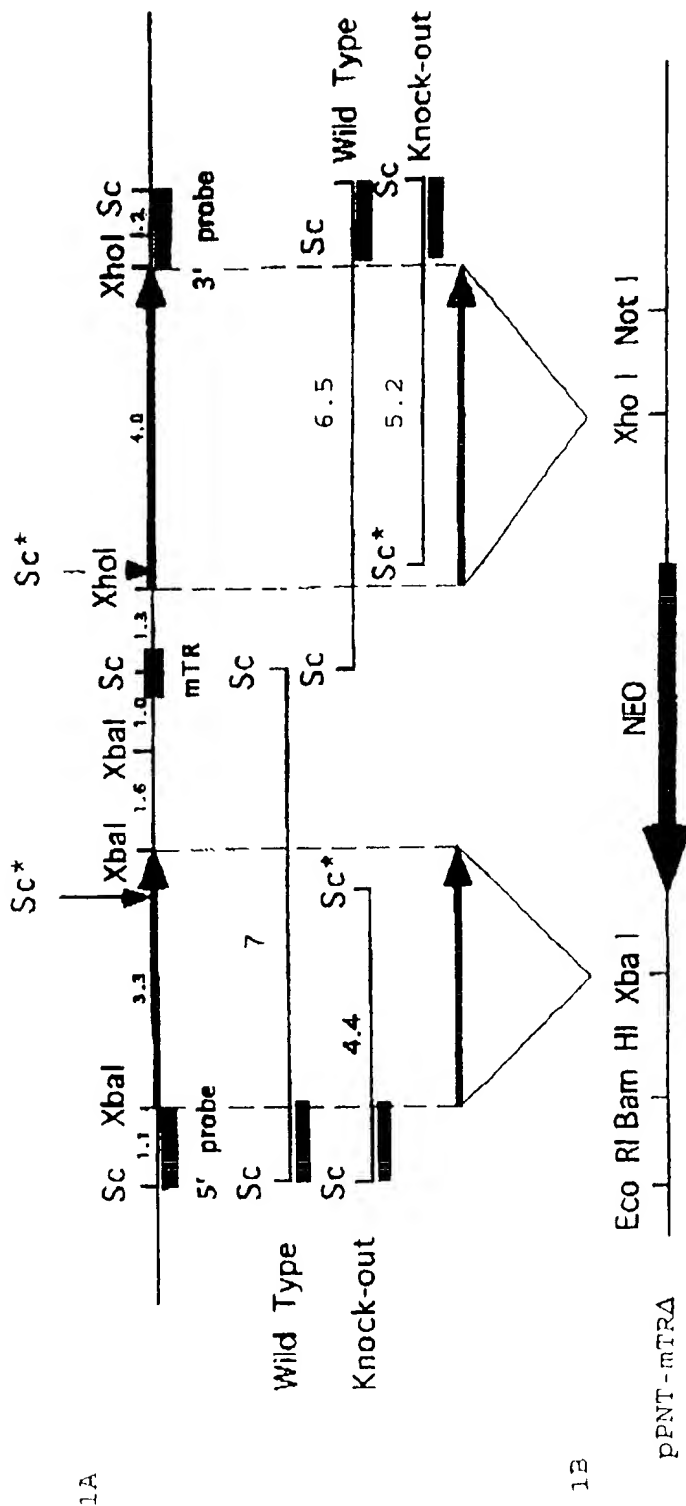
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26. A mouse embryonic stem cell selected from the group consisting of Tel-1, Tel-2, Tel-3 and Tel-4.
27. The transgenic nonhuman organism of Claim 1 wherein transcription of an endogenous telomerase RNA component sequence is under the control of a promoter sequence different from the endogenous promoter sequence controlling the transcription of the endogenous gene.
28. A knockout mouse wherein the endogenous gene is replaced in part or in whole by homologous recombination between the endogenous gene and a DNA construct comprising DNA encoding exogenous DNA between the 3.3 kb *Xba*I fragment of the 5' end of the mouse telomerase RNA component gene and the 4.0 kb *Xho*I fragment of the 3' end of the mouse telomerase RNA component gene.
29. A method of identifying an agent that stimulates telomerase activity, the method comprising:
- a) administering an agent to a transgenic nonhuman organism, wherein the organism does not express telomerase activity or has diminished telomerase activity but is capable of producing telomerase activity; and
 - b) assessing the effect of the agent on the telomerase activity,
- wherein if the agent causes an increase in telomerase activity, the agent is active in stimulating telomerase activity.
30. A method of identifying an agent that stimulates telomerase activity, the method comprising:
- a) administering an agent to a sample of transgenic

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- cells or tissue obtained from a transgenic nonhuman organism, wherein the sample does not express telomerase activity or has diminished telomerase activity but is capable of producing telomerase activity; and
- 5 b) assessing the effect of the agent on the telomerase activity,
- wherein if the agent causes an increase in telomerase activity, the agent is active in stimulating
- 10 telomerase activity.
31. A method of identifying an agent that is active in inhibiting telomerase activity, which comprises:
- a) administering an agent to a transgenic nonhuman organism, wherein the organism expresses
- 15 telomerase activity; and
- b) assessing the effect of the agent on the telomerase activity,
- wherein if the agent causes a decrease in telomerase activity, the agent is active in inhibiting telomerase
- 20 activity.
32. A method of identifying side effects of an agent that modifies telomerase activity in an organism, which comprises:
- a) administering the agent to a transgenic nonhuman organism which is incapable of expressing
- 25 telomerase; and
- b) assessing the effects of the agent on the transgenic nonhuman organism,
- wherein if the agent causes one or more effects on the
- 30 organism, it is identified as an agent that causes one or more side effects.

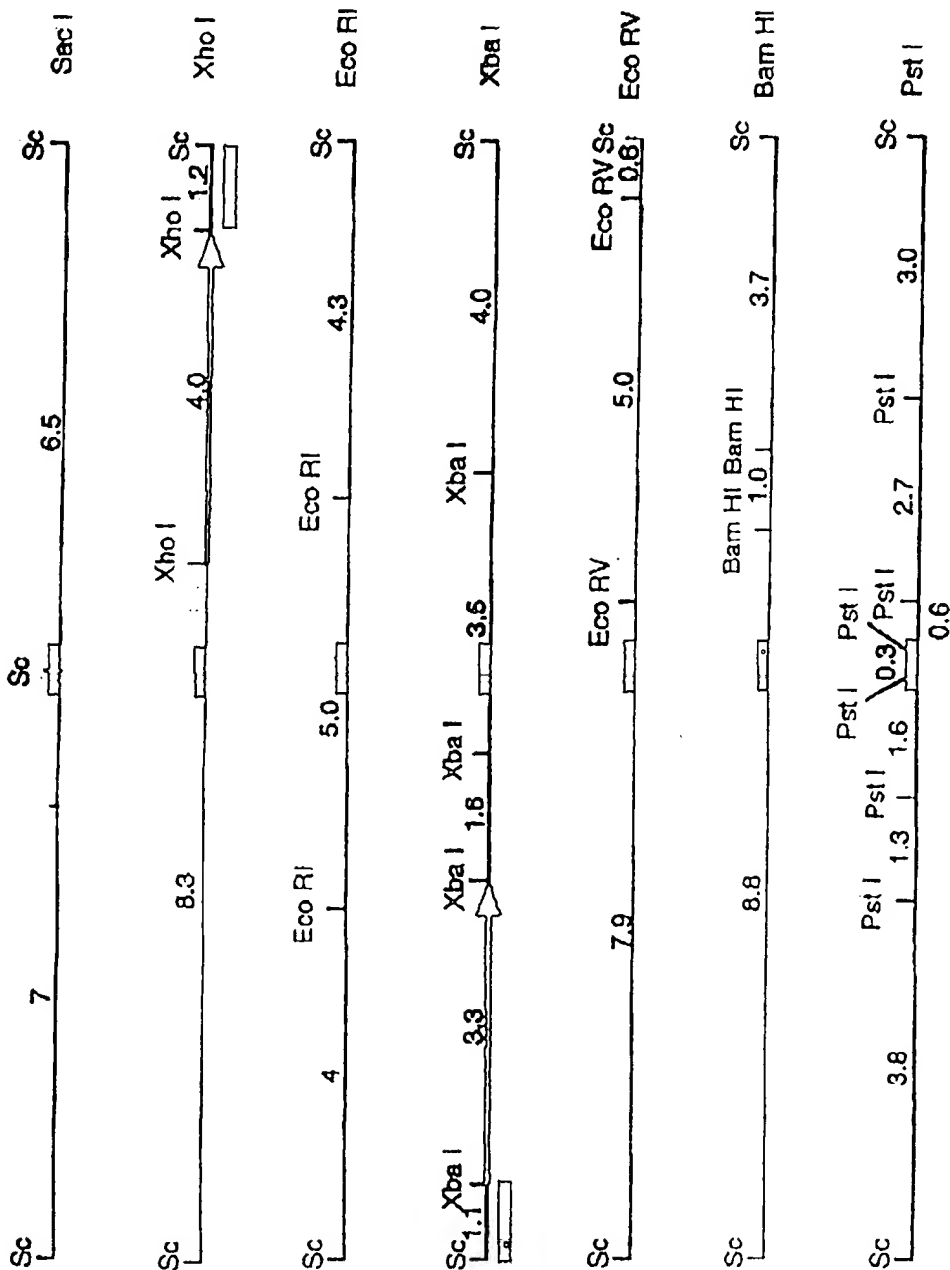
TARGETING CONSTRUCT FOR KNOCKING OUT MOUSE TELOMERASE RNA COMPONENT



* Site-directed mutagenesis

FIGURE 1A-1B

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Restriction Map of Genomic mTR gene.

FIGURE 2

CLONING OF XBA I IN SK (-)
(determine orientation)

CLONING OF XHO I IN KS (+)
(determine orientation)

SEQUENCE 3' OF XBA I FRAGMENT
(design primer for mutagenesis)

SEQUENCE 5' OF XHO I FRAGMENT
(design primer for mutagenesis)

INTRODUCE SAC I RESTRICTION SITE
IN BOTH FRAGMENTS :

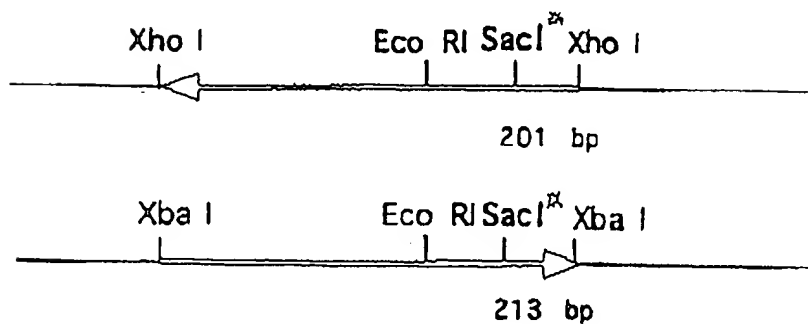


FIGURE 3

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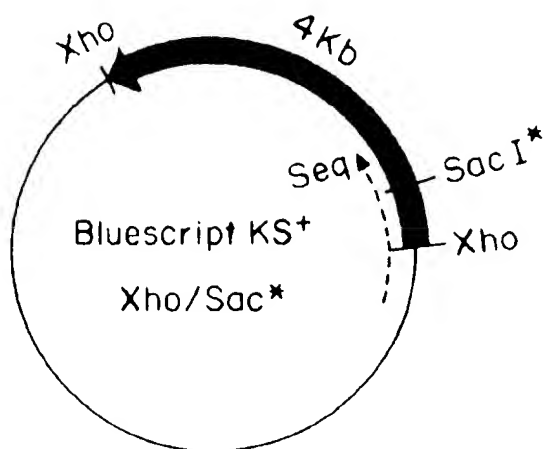


FIG. 4A

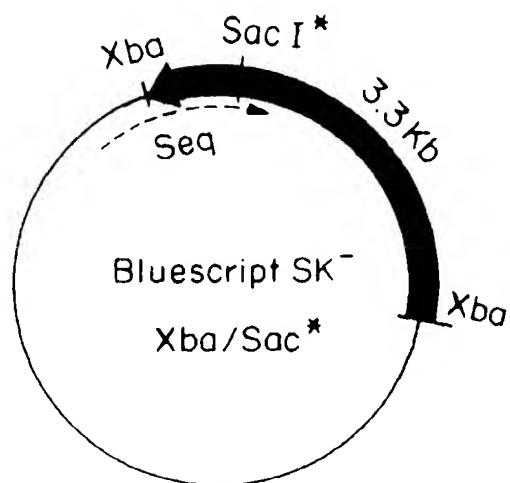


FIG. 4B

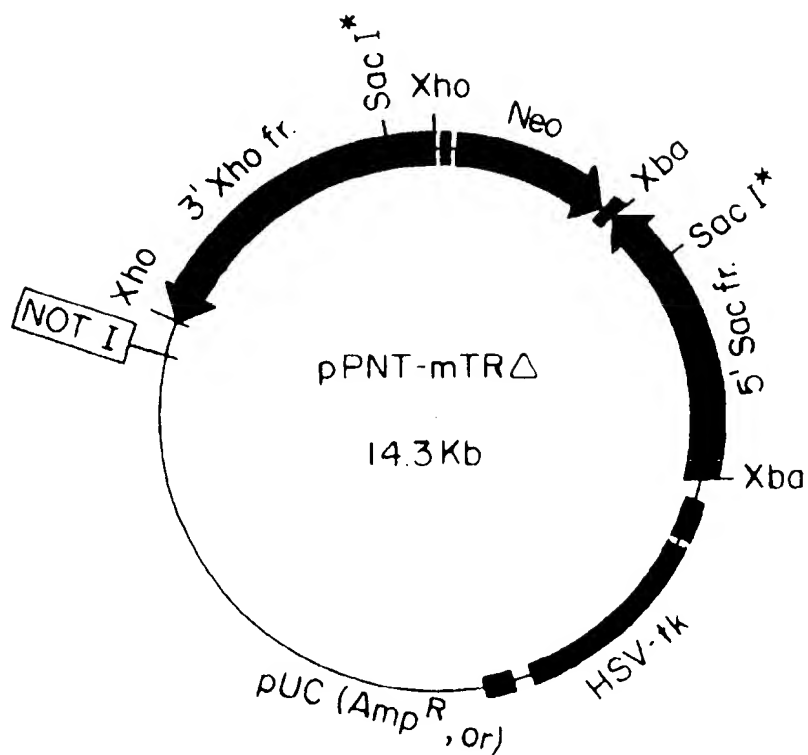


FIG. 4C

Human Telomerase RNA Component Gene

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-->ST
|
GGGTTGCGGA GGGTGGGCCT GGGAGGGGTG GTGGCCATTT TTTGTCTAAC 50
CCTAACTGAG AAGGGCGTAG GCGCCGTGCT TTTGCTCCCC GCGCGCTGTT 100
TTTCTCGCTG ACTTTCAGCG GCGGAAAAG CCTCGGCCTG CCGCCTTCCA 150
CCGTTTCATTC TAGAGCAAAC AAAAAATGTC AGCTGCTGGC CCGTTCGCCT 200
CCCGGGGACC TCGGGCGGGT CGCCTGCCCA GCGCCGAAC CCGCCTGGA 250
GCCGCGGTCTG GCGCGGGGCT TCTCCGGAGG CACCCACTGC CACCGCGAAG 300
AGTTGGGCTC TGTCAGCCGC GGGTCTCTCG GGGGCGAGGG CGAGGTTTAC 350
CGTTTCAGGC CGCAGGAAGA GGAACGGAGC GAGTCCCGCC GCGGCGCGAT 400
TCCCTGAGCT GTGGGACGTG CACCCAGGAC TCGGCTCACA CATGCAGTTC 450
GCTTTCCTGT TGGTGGGGGG AACGCCGATC GTGCGCATCC GTCACCCCTC 500
GCCGGCAGTG GGGGCTTGTG AACCCCCAAA CCTGACTGAC TGGGCCAGTG 550
TGCTGCAAAT TGGCAGGAGA CGTGAAGGCA CCTCCAAAGT CGGCCAAAAT 600
GAATGGGCAG TGAGCCGGGG TTGCCTGGAG CCGTTCCTGC GTGGGTTCTC 650
CCGTCTTCCG CTTTTTGTTG CCTTTTATGG TTGTATTACA ACTTAGTTCC 700
TGCTCTGCAG ATTTTGTTGA GGTTTTTGCT TCTCCCAAGG TAGATCTCGA 750
CCAGTCCCTC AACGGGGTGT GGGGAGAACA GTCATTTTTT TTTGAGAGAT 800
CATTTAACAT TTAATGAATA TTTAATTAGA AGATCTAAAT GAACATTGGA 850
AATTGTGTTC CTTTAATGGT CATCGGTTTA TGCCAGAGGT TAGAAGTTTC 900
TTTTTTGAAA AATTAGACCT TGGCGATGAC CTTGAGCAGT AGGATATAAC 950
CCCCACAAGC TT 962

```

FIGURE 5

Mouse Telomerase RNA Component Gene

CTCGACCAAT	CAGCGCGCGC	CATGGGGTAT	TTAAGGTCGA	GGGCGGCTAG	50
GCCTCGGCAC	<u>CTAACCCTGA</u>	TTTTCATTAG	CTGTGGGTTC	TGGTCTTTTG	100
TTCTCCGCCC	GCTGTTTTTC	TCGCTGACTT	CCAGCGGGCC	AGGAAAGTCC	150
AGACCTGCAG	CGGGCCACCC	GGCGTTCCCG	AGCCTCAAAA	ACAAACGTCA	200
GCGCAGGAGC	TCCAGGTTCG	CCGGGAGCTC	CGCGGCGCCG	GGCCGCCCAG	250
TCCCGTACCC	GCCTACAGGC	CGCGGCCCGC	CTGGGGTCTT	AGGACTCCGC	300
TGCCGCCGCG	AAGAGCTCCG	CCTCTGTCAG	CCGCGGGCGC	GCGGGGGCTG	350
GGGCCAGGCC	GGGCGAGCGC	CGCGAGGACA	GGAATGGAAC	TGGTCCCCGT	400
GTTCGGTGTC	TTACCTGAGC	TGTGGGAAGT	GCACCCGGAA	CTCGGTTCTC	450
ACAACCCCCA	TTCCCGCTGG	GGAAATGCCC	CGCTGCAGGG	CGGGCCGCTA	500
GAACCTGCGA	CTCTGGGGAA	AGGGGCTTCG	GTGTGAGACG	GTAGCCAGCC	550
AAAGGGTATA					560

FIGURE 6

+1
↓

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hTR  GCTCCCTTTATAAGCCGACTCGCCCGCAGCGCACCGGGTTGCGGAGGGA
bTR  CAGCCTTCAAAAATGAGGAGHTCCGGGTTTGGGAGGGTGGGCCCCGGGTT
cTR  GCGAGAGCCGGCGCCGCCAATCAGCGCGCGCCACC-----CC
mTR  GCAAGTGCTGGGCTCGACCAATCAGCGCGCGCCATGCGGTATTAAAGGTC
rTR  TGACTATTAGGGCTCAGCCAATCAGCGCGAGCTGTGCGGTATTAGGAC

hTR  TGGTGGGCTCGGAGGGGTTGGC---CATTTTTGTCTAACCTAA-
bTR  TGGTGGGCCC---CGGGTTGG-TGGCAGCCATTTCTCATCTAACCTAAT
cTR  TGGTACTTAAAGCGGACCTGTCGGGC-GGCT-GCCAGTCTAACCTGAAT
mTR  TGGTATTTAAGGTCGA---GTCGGCTAGGCTTCGGCACCTAACCTGAAT
rTR  TGGTATTTAGGGACAA---GTCGGCGGACTTCTGCGTCTAACCTAAT

hTR  -CTGAGAAGGCG-CTAAGCGCGGCTTTTGGGTCGCGCGCGCTGTTTTT
bTR  T---GAGACAGCG-CTAAGCGCGCTGTGCTTTTGGTTACCGCGCGCTGTTTTT
cTR  TCTGAG---AGCGTGGGCTACTGTGCTTTGG-TCTCGCGCGCTGTTTTT
mTR  TTCATT---AGCGTGGGCTTCTGCTTTTGGTTCTCGCGCGCTGTTTTT
rTR  GTTATA---GCGTGGGCTTCTGCTTTTGGTTCTCGCGCGCTGTTTTT

hTR  CTCGCTGACTTTCAGCGGGC-GAAGAGCTCGGCTTCTCGCTTCAGC
bTR  CTCGCTGACTTTCAGCGGGC-GAAGAGCTCGGCTTCTCGCTTCAGC
cTR  CTCGCTGACTTTCAGCGGGC-GAAGAGCTCGGCTTCTCGCTTCAGC
mTR  CTCGCTGACTTTCAGCGGGC-GAAGAGCTCGGCTTCTCGCTTCAGC
rTR  CTCGCTGACTTTCAGCGGGC-GAAGAGCTCGGCTTCTCGCTTCAGC

hTR  GTTCATTCTAGACAAACAAAAA---TGTGAGCTGCTGGCGTTTCGCC
bTR  ATCAGCTCTGACAAACAAAAA---TGTGAGCTGCTGGCGTTTCGCC
cTR  GCGCTTTTCCA-CCA-CAAAAAA---TGTGAGCTGCTGGCGTTTCGCC
mTR  CGGCTTCCCGAGCT-CAAAACAAAGTTCAGCGCAAGGAGCTTCAGGT
rTR  GCGCTTCTGGA-CCT-CAAAAAA---TGTGAGCTGAGGAGCTC-TGGT

hTR  CCTCCCGGGA-CCTTCGGGCGGT---CGGTGCGCAGCGCCGAACCCGCC
bTR  TCTCCCGGGAACCTTCGGGTGT---CCGCGCGCCAGCGCCAGTGCCCGGCC
cTR  T-----GGAGCCT---TGGC-CCGCGCGCCAGCGCCGCA---CCCGCC
mTR  TCGCCG-GGAGC-TCCGCGCGCGCGCGCGCGCCAGTCCCGTA---CCCGCC
rTR  GCC-----AGAGC-TCCGCGCGCTGGGCGCGCCAGCGCGTA---CCCGCC

hTR  TGGAGGCCGCGGTTCGGC-GGGCTTCTCGGAGGCACTCACTGCCAFCG
bTR  TG-AGGCCGCGGTTCGGC-GGGCTTCTCGGAGGCACTCACTGCCAFCG
cTR  TG-AGGCCGCGGTTCGGC-TGGAG---TCTCGGCTTC-GCTGCCGCGG
mTR  TACAGGCCGCGGTTCGGC-TGGAG---TCTAGGACTTC-GCTGCCGCGG
rTR  TGGAGGCCGCGGTTCGGC-TGGAG---TCTAGGACTTC-GCTGCCGCGG

hTR  CGAAGAG-TTGGGCTCTGTTCAGCCGCGGTCTCTCGGGCGGAGGTCAG
bTR  TGAAGAG-TTGGGCTCTGTTCAGCCGCGGTCTCTCGGGCGGAGGTCAG
cTR  CGAAGAG-TT-AGACTCTGTTCAGCCGCGGT-GCGTCAGGGCTTGGGTCAG
mTR  CGAAGAG-TTCCGCTCTGTTCAGCCGCGGT-GCGTCAGGGCTTGGGTCAG
rTR  TGAAGAG-TT-AGTCTCTGTTCAGCTACGGG-GCACCGGGCTTGGGTCAG

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FIGURE 7A

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hTR GTTCACCGTTTCAGGCCGC-AGGAAGAGGAACGAAACGA-ATCCC-GCG
bTR GGCTGTA-----ACCGC-AGGAAAAGGAACGAAATGGGATCCCAGCG
cTR GCCGG--CAGC-----GCCGCAACCAAGAAA-CGAGCTG-ATCCC-CTG
mTR GCCGGGCGAGC-----GCCGCGAGGACAGGAAT-AGACTG-ATCCCCTG
rTR GCCGGGAGAGC-----GCCGCAAGGACAGTAAC-AGACTG-ATCCCCTGAG

hTR CGCGCGCGGATTCTCTGAGCTATGGGNCCTGCACCAAGACTCGGCTCAG
bTR CGCG-TGG-ATCCCTGAGCTGTGGGACTGACCCGAGACTCGGCTCAG
cTR AACGGTGAC-ATCCCTGAGCTGTGGGAATGCACCAAGACTCGGCTCAG
mTR TTGGGTGTC-ATCCCTGAGCTGTGGGAGTGCACCGAGACTCGGCTCAG
rTR TTGGGTGTC-ATCCCTGAGCTGTGGGAGTGCACCGAGACTCGGCTCAG

hTR ACRTGCAGTTGCTTTCCTGTGGTGCGGAAACGCGATCGTGCGCATC
bTR ACRTCTGAAAAAAAAAAAAA-----TGAGAGAT-CCTACCATATG-AAAC
cTR ACNACCCCCAACCCCGC-----TGCGAATAACCTG-CTGCARAGCGG
mTR ACNACCCCCATTCCCGC-----TGCGAATAACCTG-CTGCARAGCGG
rTR ACNACCCCCACTTCCCGC-----TGCGAATAACCTG-CTGCARAGCGG

hTR CGTCAACCCCTCGCCGGCAGTGGGGCTTGTGAACCCCAACCTGACTGA
bTR AATATGAAC-AAACTTG-AGGTTGTGCTAAGTGAAG-TAGTC-----
cTR G-----CCCCTAGGACCTGGCAGCCCGAGGAATGGTG-CCAACGTGTGTGC
mTR G-----CCGCTAGAACCTG-CGACTCTGGGAAAGGGGCTTCGGTGTGAGA
rTR GG-----CGCTAGAAC-TG-CAACGGGAGGAACGGGGCCAAAGGTGTGTGC

hTR CTG-GGCCAGTGTGTGTC
bTR ----AGCCATAGAAGGACAAATACTGTTACAATTC
cTR ACATGCCAGAGTGGGCGATG
mTR CGGTAGCCAGCCAAAGGGTATA
rTR ACGAGGCCACGGTGCTC

```

FIGURE 7B

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Tetrahymena 80kD Telomerase Protein Component Gene

```

1  aactcattta attactaatt taatcaacaa gattgataaa aagcagtaaa taaaacccaa
61 tagattttaat ttagaaaagta tcaattgaaa aatggaaaatt gaaaaacaact aagcacaata
121 gccaaaagcc gaaaaattgt ggtgggaact tgaattagag atgcaagaaa accaaaatga
181 tatataagtt agggttaaga ttgacgatcc taagcaatat ctctgtaacg tcactgcagc
241 atgtttgttg taggaaggta gttactacta agataaagat gaaagaagat atatoatcac
301 taaagcaact cttgaggtgg ctgagttctga tcctgagttc atctgctagt tggcagtcct
361 catccgtaat gaactttaca tcagaactac cactaactac attgtagcat tttgtgttgt
421 ccacaagaat actcnaacoat tcactcgaaaa gtacttcaac aaagcagtao ttttgccata
481 tgacttaact gaagttctgtg aatttgcata ggttctctat atttttgatg caactgaatt
541 caaaaatttg tatcttgata ggatacttct ataagatatt cgttaaggaa tcactttccg
601 taagtgttta caaagatgag tcagaagcaa gttttctgaa ttcaacgaat actaacttgg
661 taagtattgc actgaatcct aacgtaagaa aacaatgttc cgttacctct cagttacca
721 caagtaaaag tgggattana ctaagaagaa gagaaaagag aatctcttaa ccaacttta
781 ggcaataaag gaatctgaag ataagtccaa gagagaaact ggagacataa tgaacgttga
841 agatgcaatc aagggtttta aaccagcagt tatgaagaaa atagccaaga gatagaatgc
901 catgaagaaa cacatgaagg cacctaaaaa tcctaactct accttggaat caaagtactt
961 gaccttcaag gatctcatta agttctgcc aattctctgag cctaaagaaa gactctataa
1021 gatccttggg aaaaaatacc ctaagaccga agaggaatac aaagcagcct ttggtgattc
1081 tgcatctgca ccottoaatc ctgaattggc tggaaagcgt atgaagattg aaatctctaa
1141 aacatgggaa aatgaactca gtgcaaaagg caaactgct gaggtttggg ataatttaat
1201 ttcaagcaat taactcccat atatggccat gttacgtaac ttgtctaaca tcttaaaagc
1261 cgggtgttca gatactacac actctattgt gatcaacaag atttgtgagc ccaaggccgt
1321 tgagaactcc aagatgttcc ctcttcaatt ctttagtgcc attgaagctg ttaatgaagc
1381 agttactaag ggattcaagg ccaagaagag agaaaatatg aatcttaaa gtc aaatcga
1441 agcagtaaa ggaagtgttg aaaaaaccga tgaagagaag aaagatatgg agttggagta
1501 aacogaagaa ggagaatttg ttaaagtcaa cgaaggaatt ggcaagcaat acattaaactc
1561 cattgaactt gcaatcaaga tagcagttta caagaattta gatgaatca aaggacacac
1621 tgcaatcttc tctgatgttt ctggttctat gagtaoctca atgtcaggtg gagccaagaa
1681 gtatgggtcc gttcgtactt gtctcgagtg tgcattagtc cttggtttga tggtaaaata
1741 acgttgtgaa aagtootcat tctacatctt cagttcacct agttctcaat gcaataagtg
1801 ttacttagaa gttgatctcc ctggagacga actcogtcc tctatgtaaa aacttttgca
1861 agagaagga aaacttggtg gtggtactga tttccctat gagtgcattg atgaatggac
1921 aaagaataaa actcacgtag acaatatcgt tattttgtct gatatgatga ttgcagaagg
1981 atattcagat atcaatgtta gaggcagttc cattgttaac agcatcaaaa agtacaagga
2041 tgaagtaaat cctaacatta aaatctttgc agttgactta gaaggttacg gaaagtgcct
2101 taatctaggt gatgagttca atgaaaacaa ctacatcaag atattcggta tgagcgattc
2161 aatcttaaa gttcatttcag ccaagcaagg aggagcaaat atggtcgaag ttatcaaaaa
2221 ctttgccctt caaaaaatag gacaaaagtg agtttcttga gattcttcta taacaaaaat
2281 ctaccccac tttttgtttt tattgcatag ccattatgaa atttaaatat ttatctattt
2341 atttaagtta cttacatagt ttatgtatcg cagttctatta gcctattcaa atgattctgc
2401 aaagaacaaa aaagattaaa a

```

FIGURE 8

SUBSTITUTE SHEET (RULE 26)

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Amino Acid Sequence of the Tetrahymena
80 kD Telomerase Protein Component

MEIENNQAQQPKAEKLWWELELEMQENQNNDIQVRVKIDDPKQYL
VNVTAACLLQEGSYQDKDERRYIITKALLEVAESDPEFICQLAVYIRNELYIRTTN
YIVAFCVVHKNTQPFIEKYFNKAVLLPNDLLEVCEFAQVLYIFDATEFKNLYLDRILS
QDIRKELTFRKCLQRCVRSKFSEFNEYQLGKYCTESQRKKTMFYLSVTNKQKWDQTK
KKRKENLLTKLQAIKESEDKSKRETGDIMNVEDAIAKALPVMKKIAKRQAMKKHMK
APKIPNSTLESKYLTFKDLIKFCHISEPKERVYKILGKKYPKTEEEYKAAFSDSASAP
FNPELAGKRMKIEISKWENELSAKGNTAEVWDNLISSNQLPYMAMLRNLSNILKAGV
SDTTHSIVINKICEPKAVENSKMFPLQFFSAIEAVNEAVTKGFKAKKRENMNKLGQIE
AVKEVVEKTDEEKDMELEQTEEGEFVKVNEGIGKQYINSIELAIAVKNLDEIKG
HTAIFSDVSGSMSTMSGGAKKYGSVRTCLECALVLGLMVKQRCEKSSFYIFSSPSSQ
CNKCYLEVDPGDELRPMSQKLLQEKGLGGTDFPYECIDEWTKNKTHTVDNIVILSD
MMIAEGYSIDINVRGSSIVNSIKKYKDEVNPNIKIFAVDLEGYGKCLNLGDEFNENNYI
KIFGMSDSILKFISAKQGGANMVEVIKNFALQKIGQK"

FIGURE 9

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Tetrahymena 95 kD Telomerase Protein Component Gene

```

1  tcaatactat taattaataa ataaaaaaaaa goaaactaca aagaaaatgt caaggcgtaa
61  ctaaaaaaag ccatagggtc ctataggcaa tgaacaaaat cttgattttg tattacaaaa
121 tctagaagtt tacaaaagcc agattgagca ttataagacc tagtagtaat agatcaaaga
181 ggaggatctc aagcttttaa agttcaaaaa ttaagattag gatggaaaot ctggcaaoga
241 tgatgatgat gaagaaaaca actcaaataa ataataagaa ttattaagga gagtcaatta
301 gattaagtag caagtttaast tgataaaaaa agttggttct aaggtagaga aagatttgaa
361 tttgaacgaa gatgaaaaca aaaaagaatgg actttotgaa tagcaagtga aagaagagta
421 attaagaacg attactgaag aatagggttaa gtattaaaat ttagtattta acatggacta
481 ccagtttagat ttaaatgaga gtggtggcca tagaagacac agaagagaaa cagattatga
541 tactgaaaaa tggtttgaaa tatctcatga ccaaaaaaat tatgtatcaa tttacgccaa
601 ctaaaagaca tcatattggt ggtgggttaa agattatttt aataaaaaa attatgatca
661 tcttaatgta agcattaaca gactagaaac tgaagccgaa ttctatgcct ttgatgattt
721 ttcacaaaca actcaactta ctaataattc ttactagact gttaacatag acgttaattt
781 tgataataat ctctgtatac tcgcattgct tagattttta ttatcactag aagatttcaa
841 tatttttgaa ataagatott ctatatacaag aaattaatat aatttttgaga aaattggtga
901 gctacttgaa actatcttcg cagtgtgtctt ttctcatcgc cacttacaag gcattcattt
961 acaagttcct tgcgaagcgt tctaataatt agttaactcc tcatcataaa ttagcgttaa
1021 agatagctaa ttatagggtat actotttctc tacagaacta aaattagttg acactaacaa
1081 agtccaagat tattttaagt tcttataaga attccctcgt ttgactcatg taagctagta
1141 ggttatccca gttagtgcta ctaacogtgt agagaacctc aatgttttac ttaaaaaggt
1201 caagcatgct aatcttaatt tagtttctat ccctacctaa ttcaattttg atttctactt
1261 tgttaattta taacatttga aattagagtt tggattagaa ccaaatattt tgacaaaaca
1321 aaagcttgaa aatctacttt tgagtataaa ataatacaaa aatcttaaat ttttaagatt
1381 aaacttttac acctacgttg cttaaagaaac ctccagaaaa cagatattaa aacaatctac
1441 aacaatcaaa aatctcaaaa acaataaaaa tcaagaagaa actcctgaaa ctaagatga
1501 aactccaagc gaaagcacaa gtggtatgaa attttttgat catctttctg aattaaccga
1561 gcttgaaagt ttcagcgtta acttgtaayc tacccaagaa atttatgata gcttgacaa
1621 acttttgatt agatcaacaa atttaagaa gttcaaatat agttacaaat atgaaatgga
1681 aaagagttaa atggatacat tcatagatct taagaatatt tatgaaacct taacaatct
1741 taaaagatgc tctgttaata tatcaaatcc tcatggaaac atttcttatg aactgacaaa
1801 taaagattct actttttata aatttaagct gaccttaaac taagaattat aacacgttaa
1861 gtatactttt aagtagaacg aattttaatt taataacgtt aaaagtgc aaattgaatc
1921 ttctctatta gaaagcttag aagatattga tagtctttgc aaatctattg cttcttgtaa
1981 aaatttacaa aatgttaata ttatcgccag ttgtctctat cccaacaata tttagaaaaa
2041 tcctttcaat aagcccaatc ttctattttt caagcaattt gaataattga aaaatttgga
2101 aaatgtatct atcaactgta ttcttgatca gcatatactt aattctattt cagaattott
2161 agaaaaaat aaaaaataa aagcattcat ttgaaaaaga tattatttat tacaatatta
2221 tcttgattat actaaattat ttaaaacact tcaatagtta cctgaattaa attaaattta
2281 cattaattag caattagaag aattgactgt gagtgaagta cataagtaag tatgggaaaa
2341 ccacaagcaa aaagctttct atgaaccatt atgtgagttt atcaaagaat catcctaaac
2401 cctttagcta atagattttg accaaaacac tgtaagtgat gactctatta aaaagatttt
2461 agaacttata tctgagtcta agtatcatca ttatttgaga ttgaacccta gttaatctag
2521 cagttaattt aaatctgaaa acgaagaaat ttaagaactt ctcaaagctt gcgacgaaaa
2581 aggtgtttta gtaaaagcat actataaatt ccctctatgt ttaccaactg gtacttatta
2641 cgattacaat tcagatagat ggtgattaat taatatattg tttaaataaa tattaatat
2701 tgaatatttc ttgcttatt atttgaataa tacatacaat agtcattttt agtgttttga
2761 atatatttta gttatttaatt tcattatttt aagtaaataa ttatttttca atcatttttt
2821 aaaaaatcg

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FIGURE 10

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Amino Acid Sequence of the *Tetrahymena*
95 kD Telomerase Protein Component

MSRRNQKKPQAPIGNETNLD FVLQNLEVYKSQIEHYKTQQQQIK
EEDLKLLKFKNQDQDGN SGNDDEENNSNKQQELLRRVNQIKQQVQLIKKVGSKVEK
DLNLNEDENKKNGLSEQQVKEEQ LRTITEEQVKYQNLVFNMDYQLDLNESGGHRRHRR
ETDYDTEKWF EISHDQKNYVSIYANQKTSYCWWLKD YFNKNNDHLNVSINRLETEAE
FYAFDDFSQTIKLTNNSYQTVNIDVNF DNNLCILALLRFLLSLERFNILNIRSSYTRN
QYNFEKIGELLE TIFAVVFSHRHLQGIHLQVPCEAFQYLVNSSSQISVKDSQLQVYSF
STDCLKLVDTNKVQDYFKFLQEF PRLTHVSQQAIPVSATNAVENLNVLLKKVKHANLNL
VSIPTQFNFD FYFVNLQHLKLEFGLEPNILTKQKLENLLLSIKQSKNLKFLRLNFYTY
VAQETSRKQILKQATTIKNLKNNKNQEETPETKDETPSESTSGMKFFDHLSELTELED
FSVNLQATQEIIYDSLHKLLIRSTNLKKFKLSYKYEMEKSKMDTFIDLKNIYETLNNLK
RCSVNISNPHGNISYELTNKDSTFYKFKLT LNQELQHAKYTFKQNEFQFNNVKSAKIE
SSSLESLEDIDSLCKSIASCKNLQNVNIIASLLYPNNIQKNPFNKPNNLLFFKQFEQLK
NLENVSINCILDQHILNSISEFLEKNKKIKAFILKRYLLQYYLDYTKLFKTLQQLPE
LNQVYINQQLEELTVSEVHKQVWENHKQKAFYEPLCEFIKESSQTLQLIDFDQNTVSD
DSIKKILESISESKYHHYLRNLNPSQSSSLIKSENEEIQELLKACDEKGVLVKAYYKFP
LCLPTGTYYDYNSDRW

FIGURE 11

GGGCGGATCCATGGAGATCGAGAACAACCAAGCTCAACAACCGAAGGCTGAGAGCTGTGG
TGGGAGCTGGAGCTGGAGATGCAAGAGAACCAAAACGACATCCAAGTTCTGTAAAGATCG
ACGACCCGAAGCAATACCTGGTTACGTTACCGCTGCTTGTCTGCTGCAAGAGGGCAGCTA
CTACCAAGACAAGGAAGAGGGTCTGTACATCATCACCAAGGCTCTGCTGGAGGTTGCTGAG
AGCGACCCGGAGTTCATCTGTCAACTGGCTGTTTACATCCGTAACGAGCTGTACATCCGTA
CCACCAACAATACATCGTTGCTTTCTGTGTTGTTTACACAAGAACAACCAACCGTTTCATCGA
GAAGTACTTCAACAAGGCTGTTCTGCTGCCGAACGACCTGCTGGAGGTTTGTGAGTTCCGT
CAAGTTCTGTACATCTTCGACGGTACCGAGTTCAAGAACCTGTACCTGGACCGTATCCTGA
GCCAAGATATCCGTAAGGAGCTGACCTTCCGTAAGTGTCTGCAACGTTGTGTTCTGTAGCAA
GTTTCAGCGAGTTCAACGAGTACCAACTGGGCAAGTACTGTACCGAGAGCCAACGTAAGAAG
ACCATGTTCCGTTACCTGAGCGTTACCAACAAGCAARAGTGGGACCAACCAAGAAGAAGC
GTAAGGAGAACCTGCTGACCAAGCTGCAAGCTATCAAGGAGAGCGAGGACAGAGCAAGCG
TGAGACCCGGGACATCATGAACGTTGAGGAGCGCTATCAAGGCTGTGAAGCCGGCGGTTATG
AAGAAGATCGCTAAGCGTCAAAACGCTATGAAGAAGCACATGAAGGCTCCGAAGATCCCGA
ACAGCACCCCTGGAGAGCAAGTACCTGACCTTCAAGGACCTGATCAAGTTCTGTACATCAG
CGAGCCGAAGGAACGTGTTTACAAGATCCTGGGCAAGAAGTACCCGAAGACCGAGGAGGAG
TACAAGGCTGCTTTTCCGGCAGACGCGCTAGCGCTCCGTTCAACCCGGAGCTGGCTGGCAAGC
GTATGAAGATCGAGATCAGCAAGACCTGGGAGAACGAGCTGAGCGCTAAGGGCAACACCGC
TGAGGTTTGGGACAACCTGATCAGCAGCAACCAACTGCCGTACATGGCCATGCTGCGTAAC
CTGAGCAACATCCTGAAGGCTGCGGTTAGCGACACCACCCACAGCATCGTTATCAACAAGA
TCTGTGAGCGGAAGGCTGTTGAGAACAGCAAGATGTTCCCGCTGCAATTCTTCAGCGCTAT
CGAGGCTGTTAACGAGGCGGTTACCAAGGGCTTCAAGGCTAAGAAGCGTGAGAACATGAAC
CTGAAGGGCCAAATCGAGGCTGTTAAGGAGGTTGTTGAGAAGACCGACGAGGAGAAGAAGG
ACATGGAGCTGGAGCAAACCGAGGAGGGCGAATTCTGTTAAGGTTAACGAGGGCATCGGCAA
GCAATACATCAACAGCATCGAGCTGGCTATCAAGATCGCTGTGAACAAGAACCTGGACGAG
ATCAAGGGCCACACCGCTATCTTCAGCGACGTCAGCGGCAGCATGAGCACCAGCATGAGCG
GCGGCGCTAAGAAGTACGGCAGCGTTCTGCTACCTGTCTGGAGTGTGCTCTGGTTCTGGGCT
GATGGTTAAGCAACGTTGTGAGAAGAGCAGCTTCTACATCTTCAGCAGCCCGAGCAGOCAA
TGTAACAAGTGTACCTGGAGGTTGACCTGCCGGGCGACGAGCTGCGTCCGAGCATGCAAA
AGCTGCTGCAAGAGAAGGGCAAGCTGGGCGGGCGGACCGACTTCCCGTACGAGTGTATCGA
TGAGTGGACCAAGAACAAGACCCACGTTGACAACATCGTTATCCTGAGCGACATGATGATC
GCTGAGGGCTACAGCGACATCAACGTTCTGTTGAGCAGCATCGTTAACAGCATCAAGAAGT
ACAAGGACGAGGTTAACCCGAACATCAAAATCTTCGCTGTTGACCTGGAGGGCTACGGCAA
GTGCTGAACCTGGGCGACGAGTTCAACGAGAACAACTACATCAAAATCTTCGGCATGAGC
GACAGCATCCTGAAGTTTCATCAGCGCTAAGCAAGGCGGCGCTAACATGGTGGAGGTGATCA
AGAATTCGCTCTGCAAAAGATCGGCCAAAGTGACTGCAGACTAGTCTAGAAAGCTTGGT
ACCGCC

FIGURE 12

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GGGGCCATGGATGAGCCGTCGTAACCAABAGAAGCCGCAAGCTCCGATCGGCAACGAGACC
AACCTGGACTTCGTTCTGCAAAACCTGGAGGTTTACAAGAGCCAAATCGAGCACTACAAGA
CCCAACAACAACAAATCAAGGAGGAGGACCTGANGCTGCTGAAGTTCAAGAACCAAGACCA
AGACGGCAACAGCGGCCAACGACGACGACGAGGAGGAGAACAACAGCAACAAGCAACAAGAG
CTGCTGCGCTGCTGTTAACCANAATCAAGCAACAAGTTCAACTGATCAAGAAGGTTGGCAGCA
AGGTTGAGAAGGACCTGAACCTGAACGAGGACGAGAACAAAGAAGAACGGCCTGAGCGAGCA
ACAAGTTAAGGAGGAGCAACTGCGTACCATCACCGAGGAGCAAGTTAAGTACCAAAAACCTG
GTTTTCAACATGGACTACCAACTGGACCTGAACGAGAGCGGCGGCCACCGTCGTCACCGTC
GCGAGACCGACTACGACACCGAGAAGTGGTTTCGAGATCAGCCACGACCAAAAGAATACGT
TAGCATCTACGCTAACCAAAAGACCAGCTACTGTTGGTGGCTGAAGGACTACTTCAACAAG
AACAACCTAGGACCACCTGAACGTTAGCATCAACCGTCTGGAGACCAGGCTGAGTTCTACG
CTTTCGACGACTTCAGCCAACCATCAAGCTGAACACAACAGCTACCAAAACCGTTAACAT
CGACGTCACCTTCGACAACAACCTGTGTATCTCTGCTCTGCTGCGTTTCTCTGCTGAGCGTG
GAGCGTTTCAACATCTGAACATCCGTAGCAGCTACACCCGTAACCAATACAACCTTCGAAA
AGATCGGCGAGCTGCTGGAGAACATCTTCGCTGTGTTTTCAGCCACCGTCACCTGCAAGG
CATCCAACCTGCAAGTTCCGCTGTGAGGCTTTTCAATACCTGGTTAACAGCAGCAGCCAAATC
AGCGTTAAGGACAGCCAACTGCAAGTTTACAGCTTCAGCACCGACCTGAAGCTGGTTGACA
CCAACAAGCTTCAAGACTACTTCAAGTTCTGCAAGAGTTCCCGCGTCTGAOCCACGTGAG
CCAACAAGCTATCCCGGTTAGCGCTACCAACGCTGTTGAGAACCTGAACGTTCTGCTGAAG
AAGGTTAAGCAGCTAACCTGAACCTGGTTAGCATCCCGACCCAAATTCAACTTCGACTTCT
ACTTCGTTAACCTGCAACACCTGAAGCTGGAGTTCCGGCTGGAGCCGAACATCCTGACCAA
GCAAAAGCTGGAGAACCTGCTGCTGAGCATCAAGCAAAGCAAGAACCTGAAGTTCTGCGT
CTGAACCTTCTACACCTACGTTGCTCAAGAGACCAGCCGTAAGCAAATCCTGAAGCAAGCTA
CCACCATCAAGAACCTGAAGAACAACAAGAACAAGAGGAGACTCCGGAGACCAAGGACGA
GACCCCGAGCGAGAGCACCAGCGCATGAAGTTCTTCGACCACCTGAGCGAGCTGACCGAG
CTGGAGGACTTCAGCGTTAACCTGCAAGCTACCCAAGAGATCTACGACAGCCTGCACAAGC
TGCTGATCCGTAGCACCAACCTGAAGAAGTTCAAGCTGAGCTACAAGTACGAGATGGAGAA
GAGCAAGATGGACACCTTCATCGATCTGAAGAATCTACGAGACCTGAACAACCTGAAG
CGTTGTAGCGTTAACATCAGCAACCCGCAACGCAACATCAGCTACGAGCTGACCAACAAGG
ACAGCACCTTCTACAAGTTCAAGCTGACCCCTGAACCAAGAGCTGCAACACGCTAAGTACAC
CTTCAAGCAAAACGAATTCCAATTCACAACGTTAAGAGCGCTAAGATCGAGAGCAGCAGC
CTGGAGAGCCTGGAGGACATCGACAGCCTGTGTAAGAGCATCGCCAGCTGTAAGAACCTGC
AAAACGTTAACATCATCGCTAGCCTGCTGTACCGAACAACATCCAAAAGAACCCGTTCAA
CAAGCCGAACCTGCTGTTCTTCAAGCAATTCGAGCAACTGAAGAACCTGGAGAACGTTAGC
ATCAACTGTATCCTGGACCAACACATCCTGAACAGCATCAGCGAGTTCTTGGAGAAGACA
AGAAGATCAAGGCTTTTATCCTGAAGCGTTACTACCTGCTGCAATACTACCTGGACTACAC
CAAGCTGTTCAAGACCCCTGCAACAACCTGCCGAGCTGAACCAAGTTTACATCAACCAACAA
CTGGAGGAGCTGACCGTTAGCGAGGTTTCAAGCAAGTTTGGGAGAACCACAAGCAAAAGG
CCTTCTACGAGCCGCTGTGTGAGTTTCAATCAAGGAGAGCAGCCAAACCTGCAACTGATCGA
CTTOGACCAAAAACACCGTTAGCGACGACAGCATCAAGAAGATCCTGGAGAGCATCAGCGAG
AGCAAGTACCACCACTACCTGCGTCTGAACCCGAGCCAAAGCAGCAGCCTGATCAAGAGCG
AGAACGAGGAGATCCAAGAGCTGCTGAAGGCTTGTGACGAGAAGGGCGTTCTGGTTAAGGC
TTACTACAAGTTCCCGCTGTGTCTGCCGACCGGCACCTACTACGACTACAACAGCGACCGT
TGGTGAGAGCTCCACCGCGGTGGCGGCCGCTCTAGAAGTAGTCCCGGAAGCTTGGGG

FIGURE 13